

STANDARD OPERATING PROCEDURE FOR TESTING WASTEWATER, FAECAL SLUDGE AND BIOSOLIDS



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Abbreviations and acronyms

BOD	Biochemical oxygen demand
Cd	Cadmium
COD	Chemical oxygen demand
CFU	Colony forming unit
CSE	Centre for Science and Environment
DAL	Double agar layer
DO	Dissolved oxygen
DWWT	Decentralized wastewater treatment system
<i>E. coli</i>	Escherichia coli
Fe	Iron
FS	Faecal sludge
FSM	Faecal sludge management
FSTP	Faecal sludge treatment plant
H. eggs	Helminth eggs
ICP-OES	Inductively Coupled Plasma Optical Emission spectroscopy
LIA	Lysine iron agar
LTB	Lauryl tryptose broth
MBBR	Moving bed biofilm reactor
MPN	Most probable number
NH ₄ ⁺	Ammonium
Ni	Nickel
Pb	Lead
PFU	Plaque forming unit
PO ₄	Phosphate
QA/QC	Quality assurance/quality control
rpm	Revolutions per minute
Std	Standard
TKN	Total Kjeldahl Nitrogen
TN	Total nitrogen
TP	Total phosphorus
TS	Total solid
TSS	Total suspended solid
VFA	Volatile fatty acid
VS	Volatile solid
VSS	Volatile suspended solid
WWTP	Wastewater treatment plant
Zn	Zinc

Preface

In the development of sustainable waste management practices, the accurate assessment of faecal sludge quality is highly essential. In the area of sanitation practices, there exists a conspicuous gap in the literature concerning the thorough characterization of faecal sludge (FS). Recognizing this dearth, this comprehensive guide aims to fill this gap by a compilation of Standard Operating Procedures (SOPs) meticulously designed for testing and analysing faecal sludge and wastewater. Characteristics of faecal sludge can be different than these other matrices by orders of magnitude. Sampling is a crucial aspect of monitoring the quality of sanitation systems, there is currently no standard method for sampling that can be used universally. As a result, researchers and practitioners need to carefully design and implement sampling strategies that are appropriate for the specific context and environment. This book consists of wide range of testing methodologies, from assessing Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), heavy metals and elemental analysis and also conducting precise microbial examinations for faecal coliforms, *E. coli*, *Salmonella* and helminths.

This guide document bridges the knowledge gap by offering step-by-step procedures and insights. Each protocol is outlined with precision, offering guidance to ensure accuracy and reliability in testing procedures for faecal sludge and/or wastewater and characterization of biosolids, which can be used as a sustainable resource for resource recovery, particularly for agriculture and soil amendment. This document aims to streamline testing processes, enhance quality control, and ultimately contribute to informed decision-making in faecal sludge management strategies. It serves as a tool for laboratory technicians, environmental scientists, sanitation practitioners, and all stakeholders invested in the effective management and treatment of faecal sludge and FS-based biosolids.

Chapter 1

1.0 Introduction

In October 2014, the Government of India (GOI) initiated the Swachh Bharat Mission with the aim of eliminating open defecation in both rural and urban areas of the country. Building restrooms for the safe containment of faecal sludge (FS) and the treatment of the sludge through treatment facilities to safeguard public health and the environment was the main aim of the project. The goal was to eradicate the hazardous practice of open defecation by promoting cleanliness, hygiene and improved solid and liquid waste management.

Faecal sludge management (FSM) is a management system that safely collects, transports and treats faecal sludge (also called septage) from pit latrines, septic tanks or other on-site sanitation facilities. Selecting the most suitable faecal sludge treatment technology is not only about providing the best technology at the lowest cost, but also about sustainability, including social and environmental acceptance, and institutional feasibility. Faecal sludge is an offensive material that contains pathogens, can generate odours and cause surface water as well as groundwater pollution. It is raw or partially digested, slurry or semisolid, the collection, storage or treatment of combinations of excreta and black water, with or without grey water. It is the solid or settled contents of pit latrines and septic tanks. It differs from sludge produced in municipal wastewater treatment plants.

Faecal sludge characteristics can differ widely from household to household, city to city, and country to country. The physical, chemical and biological qualities of faecal sludge are influenced by the duration of storage, temperature and soil condition, and intrusion of groundwater or surface water in septic tanks or pits, performance of septic tanks, and tank-emptying technology and pattern. Characteristics of faecal sludge may vary widely due to climate, toilet type, diet and other variables. Performing a waste characterization study to understand local conditions provides data that factor into treatment plant sizing as well as estimating the value of the products that can be derived from the treatment process.

1.1 BACKGROUND

The Centre for Science and Environment (CSE) is a public-interest research and advocacy organization based in New Delhi. CSE researches into, lobbies for and communicates the urgency of development that is both sustainable and equitable.

CSE has for the past few years advocated septage and decentralized wastewater treatment that can be undertaken by institutions and individuals for sustainable water and wastewater management. With the aim to push forward viable solutions, CSE is setting up a state-of-the-art referral laboratory for faecal sludge management (FSM). The lab will characterize wastewater, faecal sludge and biosolids (dried FS)—it will be the first in India to do so—to compare the performance (in terms of effective treatment) of various available on-site natural sanitation technologies.

This standard operating procedure (SOP) manual compiles testing protocols for various parameters of wastewater, faecal sludge and biosolid characterization. The selected protocols are validated experimentally in the FSM lab.

1.2 GLOBAL RELEVANCE OF ON-SITE SANITATION TECHNOLOGIES

On-site sanitation systems currently meet the demand of 2.7 billion people globally, a figure that is projected to increase to 5 billion by 2030. Sewers are connected to only a portion of metropolitan business centres. However, throughout Latin America the majority of homes in medium-sized and small towns are supplied by on-site sanitation systems (OSS), most notably septic tanks, while more than 50 per cent of homes in cities are connected to a sewerage system. Peri-urban regions in high-income nations are also home to many OSS. For instance, 25 per cent of homes in the US are equipped with septic tanks.

1.3 TYPES OF TREATMENT TECHNOLOGY

There are a variety of on-site faecal sludge treatment technologies adopted in India to treat faecal sludge and septage. The treatment efficiency of the different technologies depends upon various external factors. The geography, food culture, climatic conditions, and operation and maintenance (O&M) are important factors that affect treatment efficiency.

Taking into consideration Indian conditions, CSE has selected and evaluated the following five FSTP technologies for performance assessment:

1.3.1 Decentralized wastewater treatment system

Decentralized wastewater treatment system (DWWTS/DEWATS) is a technical approach to decentralized, community-level wastewater treatment in developing communities. The passive design uses physical and biological treatment mechanisms such as sedimentation, floatation, and aerobic and anaerobic treatment to treat both domestic and industrial wastewater sources. DEWATS is designed to be affordable, low maintenance, use local materials, and meet environmental

laws and regulations. DEWATS has service packages available for the sanitation needs of small and medium-sized enterprises, including communities, schools, municipalities, agro-industry, emergency settlements, hospitals, hotels, and prisons. DEWATS technology is also commonly used for the treatment of liquid effluent/leachate after solid–liquid separation in a FS treatment plant.

In a typical DWWTS technology, the treatment takes place in three units—settler, anaerobic baffled reactor (ABR)/anaerobic filter (AF), and planted gravel filter (PGF) bed. The settler and ABR/AF are underground units, while the PGF is constructed over the surface.

Settler: A settler is usually two-chambered and helps in the sedimentation of solids present in the wastewater (primary treatment). Wastewater enters a settler, which offers enough time for the solids to settle down due to gravity.

Anaerobic baffled reactor (ABR): The ABR provides secondary treatment for the effluent from the settler. ABR is typically five-chambered and contains filter materials (gravels and pebbles) in the last two chambers. Wastewater flows from one chamber to the other while suspended organic matter settles down in each chamber, forming a sludge blanket at the bottom where they undergo anaerobic degradation with the help of inherent microorganisms. The filter in the last two chambers prevents sludge from leaving the ABR. As time passes, this settled sludge gets activated and helps in further bio-degradation.

Planted gravel filter bed (PGF): The partially treated wastewater passes through the PGF for secondary/tertiary treatment. The PGF consists of crushed stones, pebbles and wetland plants *Canna indica* and *Typha*. These wetland plants offer further treatment by removing nutrients such as phosphates and nitrates from the wastewater.

Storage tank: The treated wastewater is stored in a storage tank from where it is used for horticulture purposes within the premises of the plant. Sometimes, the wastewater from PGF enters a polishing pond for further treatment where removal of odours and pathogens takes place by aeration and sunlight.

1.3.2 Geotube and geobag

This technology is based on the use of geotextiles—permeable fabrics made of fibre-woven polypropylene that have the potential to dewater sludge more efficiently than drying beds. Geotextiles are commercially available in a tube or bag form known as geotube or geobag and have been used in many parts of the world for

dewatering of sludge from various sources (such as in wastewater treatment and aquaculture) at different scales.

The operation is as follows:

1. Faecal sludge is flocculated by adding polymers; the flocculated sludge is filled in the geobag for treatment.
2. The free water gets drained through the permeable geotextile material within hours or days, leaving behind the dewatered sludge, which is later removed by cutting open the geobag and dried.
3. After solid–liquid separation, the solids are allowed to stay in the geobag for a few weeks for stabilization as well as moisture and pathogen reduction, which are achieved during the composting process inside the geobag.
4. Tertiary treatment like pressure sand filter, activated carbon filter and UV treatment is generally applied to the geobag effluent for final treatment before reuse/disposal. The operation of these bags is based on gravity and requires no mechanical units. The system is modular in operation, therefore making the bags potentially suitable for decentralized faecal sludge treatment. Geobags are claimed to remove TSS by >99 per cent after solid–liquid separation; TS of up to 25–30 per cent remains in the dewatered sludge. The major disadvantage of geobags is its non-reusability. These bags have to be usually discarded once the entire volume is occupied by solids.

1.3.3 Moving bed biofilm reactor

The moving bed biofilm reactor (MBBR) technology was developed in Norway at the Norwegian University of Science and Technology in cooperation with a Norwegian company, Kaldnes Miljø teknologi (now Anox Kaldnes AS) in the late 1980s. Since then, it has been widely used, with more than 700 installations throughout the world. Like SBR, MBBR technology is an advanced variation of the AS process that combines the benefits of two technologies, the activated sludge (suspended biomass) process and conventional fixed film/trickling filters (attached biomass) systems for improved treatment. These systems can be used for municipal and industrial wastewater treatment, aquaculture, potable water denitrification, and in roughing, secondary, tertiary, and side stream applications.

Technology description

The MBBR process typically includes a submerged biofilm reactor and liquid–solid separation unit. It can be operated as a 2- (anoxic) or 3- (aerobic) phase system with buoyant free-moving plastic biofilm carriers (media) that require energy (aeration or mechanical mixing) for uniform distribution throughout the bulk phase. The media used in MBBR provides increased surface area for the

microorganisms to attach to and grow in the reactors/tanks, thereby increasing the amount of microorganisms available to treat the wastewater (organic material). The increased surface area of media also reduces the footprint of the tanks required to treat the wastewater.

The biomass in the MBBR exists in two forms: suspended flocs and a biofilm attached to carriers, thus providing dual treatment. A biofilm reactor with a length-to-width ratio (L: W) in the range of 0.5:1 to 1.5:1 is optimum for an MBBR operation. An MBBR may be a single reactor or configured as several reactors in series. The media volume is up to 67 per cent of the empty bed liquid volume (or carrier fill) of an MBBR reactor. Media retaining screens are installed with one MBBR wall that retains media and allows treated effluent to flow through to the next treatment step.

Two variants exist in MBBRs depending on function/energy generation: aerobic and denitrification. In aerobic MBBRs, a diffused aeration system is used to uniformly distribute the media and meet process oxygen requirements. In denitrification MBBRs, media are distributed by mechanical mixers. Biofilm thickness on the media is controlled by airflow or mechanical mixing energy.

1.3.4 Electrocoagulation flotation

Electrocoagulation flotation (ECF) is a type of treatment technology in which electrical current is applied to flocculate and treat contaminants. Hence, the addition of flocculating agents/coagulants is not required. Direct current is applied due to which smaller particles start moving, forming aggregates which either settle or float and are later removed. The apparatus consists of a series of electrodes in the form of metal sheets arranged in pairs (positively charged anode and negatively charged cathode).

Electric current is applied as a result of which the cathode loses electrons and becomes oxidized while water gains electrons and becomes reduced. When wastewater comes in contact with a cathode electrode, metal is released and neutralizes the particulates by forming hydroxide complexes. The neutralized particles agglomerate and settle to the bottom of the tank which can be removed by filtration. In an electrocoagulation- flotation apparatus, the particulates will float to the top of the tank by means of hydrogen bubbles created at the anode. The floated particulates can be skimmed from the top of the tank. The removal efficiency of electrocoagulation process depends upon the operational parameters such as initial pH of wastewater, pollutant concentration, current density, treatment time, electrode geometry, electrode type (aluminium, steel and iron) and distance between the electrodes.

1.3.5 Packaged sewage treatment plant

Packaged sewage treatment plant (PSTP) is housed in specially designed and highly durable fibreglass-reinforced plastic (FRP) tank. These tanks are compact and can be utilized in a decentralized manner at any location, either underground or above the ground. Moving bed biofilm reactor (MBBR) technology is used in this system. This technology is designed to treat municipal sewage water, but is also used to treat leachate water after solid–liquid separation in a FSTP.

Technology description

The treatment in this system takes place in three stages—preliminary, secondary and tertiary treatments.

Preliminary treatment: A bar screen is placed to remove large inert solids such as plastic, cloth etc. from the water stream.

Secondary treatment: This happens in four distinct zones which are arranged sequentially each with a unique environment and function.

- **Anaerobic zone:** This is the first oxygen-depleted anaerobic zone in the PSTP in which the solids in raw sewage get settled while allowing the scum to float on the surface. In this zone, the settled sludge is stabilized by anaerobic digestion.
- **Anoxic zone:** The second zone is the anoxic zone with low oxygen concentration (0.2–0.5 mg/l) which is accomplished by returning the activated sludge from the final sedimentation zone. The low oxygen conditions prevailing in this zone favour the denitrification process to occur in the wastewater stream. Denitrification is carried out by anoxic microbes which break down the existing nitrates into nitrogen gas which is released into the atmosphere.
- **Aeration zone:** The third zone is the aeration zone where aerobic degradation of organic matter takes place with the help of aerobic microorganisms. In this zone, aeration is provided with the help of air blowers which accelerate the growth and activity of microbes present throughout the surface of floating plastic media (as a biofilm) added in the aeration zone. The MBBR media provides an extended surface area for the growth of microorganisms.
- **Sedimentation zone:** The final step in the secondary treatment is the settling of organic waste which occurs in the sedimentation zone. The settled waste in the bottom of the tank is pumped back to the anoxic zone for further treatment.

Tertiary treatment: The treated sewage from secondary treatment is passed through the pressure sand filter and activated carbon filter for the removal of suspended solids (TSS), colour and odour. The treated water is then disinfected using sodium hypochlorite before it is reused.

1.4 BIOSOLIDS (DRY FAECAL SLUDGE)

Faecal sludge treatment technology results in end products (biosolids) which need to be further treated, disposed of, or utilized for some type of resource recovery. For example, dried or partially dried sludge, compost, leachate and biogas are some of the end products produced from FS treatment, each of which has an inherent value, which can turn treatment from simply being a method for environmental and public health protection to resource recovery and value creation.

The various types of end products produced from different FS treatment technologies, potential difficulties and restrictions associated with their end use, and additional steps that can or should be applied to turn them into a valuable asset. The most common resource recovery approach from FS from ancient times has been its use as a soil conditioner and organic fertilizer. This is due to the presence of essential plant nutrients and organic matter in FS that increases the fertility and water-retaining capacity of soils. However, there are several other treatment options that allow for resource recovery. For example, biogas can be produced during the anaerobic digestion of FS, with the remaining sludge also being used as a soil conditioner. Novel developments are underway to recover end products as a biofuel, for example, pyrolysis, gasification, incineration and co-combustion or as resource recovery of organic matter through the growth of black soldier flies for protein production.

However, raw faecal sludge cannot be directly applied to soils, as it contains pathogenic microorganisms that create a health risk to farmers and other people who depend on the crops grown in the soil supplemented with faecal sludge. In order to reduce the microbial content, a post-treatment step for deactivating the microbes is essential. Co-composting with carbon-rich materials such as agricultural wastes, and food waste is one of the approaches to reduce the microbes as it provides two benefits: enables thermophilic temperature required for pathogen destruction and prevents nitrogen volatilization.

The biosolid contains large amounts of microorganisms, mainly originating from the faeces. These microorganisms can be pathogenic, and exposure to untreated biosolids constitutes a significant health risk to humans, either through direct contact or indirect exposure. The biosolid needs to be treated to an adequate hygienic level based on the end-use or disposal option. For instance, exposure pathways are very different for treated sludge discharged to the environment, used in agriculture, or combusted as fuel. Mechanisms for pathogen reduction and/or inactivation include starvation, predation, exclusion, desiccation, partitioning and temperature. Biosolid contains significant concentrations of nutrients, which

can be harnessed for beneficial resource recovery, but if not properly managed can result in environmental contamination. The nutrients in FS can supplement synthetic nitrogen-based fertilizers that are heavily dependent on fossil fuels and phosphorus, which is a mined resource of finite supplies.

1.5 AIM AND OBJECTIVES OF THE TESTING PROTOCOL

There is very limited knowledge about the quality or characteristics of faecal sludge in various containment systems or septic tanks. While several types of on-site sanitation systems exist in India, the performance efficiency data of the various on-site sanitation systems and treatment technologies (FSTPs) is limited or not easily accessible. There is neither any available standard protocol for testing the characterization of faecal sludge nor any institutional mechanism for testing faecal sludge in India.

India lacks science-based evidence to help make appropriate public investment decisions to address the sanitation challenges of non-sewered areas/on-site sanitation. Other regulatory measures are also non-existent or are ineffectively enforced, resulting in poor system performance, public health threats and degradation of surface- and groundwater. There is hence a direct need to build knowledge and practice decision-making and analysis of cost-effective and sustainable FS treatment technologies.

Aim

The aim of this SOP manual is to develop and validate protocols for planning, designing and implementing sustainable and affordable wastewater treatment, faecal sludge/septage management as well as biosolid reuse and resource recovery solutions.

Scope

- To develop testing protocols, process input–output sample analysis and method validation to test wastewater and faecal sludge
- To test the effectiveness of technologies in terms of performance and feasibility
- To understand how operational factors, impact the variability of faecal sludge characteristics

Chapter 2

2.0 Sampling

Sampling is the first and perhaps the most critical area of the entire process of obtaining sludge quality information. Standardized methods for sampling faecal sludge do not exist. This has likely contributed to the high data variability that has been observed in previously conducted characterization studies. It also reduces the comparability of results, limits the understanding of the faecal sludge that will be delivered to treatment facilities and prevents the optimized design of treatment plants. Proper sampling is an integral part of monitoring the quality of sludge being removed for use or disposal.

2.1 PREPARE A SAMPLING PLAN

The key elements of a sampling plan can be divided into four groups, focusing on consistency, communication, documentation and data handling. Consistency entails the assurance that samples are taken the same way from the same location for every sampling event. Communication entails making sure the lab understands the proper methods to run, target reporting level and key details regarding the facility. Proper sampling activity documentation includes proper sample labelling, chain-of-custody procedures and a log book of sampling activities. Data handling means that after all aspects of the sampling event are documented, the data is reviewed before it is submitted.

2.2 REPRESENTATIVE SAMPLES

A sample that is representative of the sludge being removed must be acquired in a manner that will not compromise its subsequent analysis. Sampling needs may vary depending upon site location, sample composition, logistics, time of collection and analytes to be measured. A single approach to sampling is neither possible nor appropriate. Grab or composite samples may be appropriate depending on what the sample is being analysed for and what the operator thinks is representative. A grab sample is a specific quantity of sludge collected at a specific time and location. A single grab sample can represent sludge quality at the time and place it was collected. A composite sample is many grab samples that have been collected and mixed together to form a single sample. Generally, composite sampling is accomplished by collecting samples of equal size. In the case of continuous processes, the time interval between grab samples is typically kept

constant. In composite sampling, the grab samples that comprise the composite should be completely and thoroughly mixed.

2.3 SAMPLE SIZE

Analytical protocols require minimum sample sizes to ensure analytical accuracy and precision. Laboratories should be consulted well in advance of any actual sample collection activities to ascertain the minimum sample size needed for each analytical method. To arrive at a statistically significant outcome, the sample size should ideally be big enough and randomized.

2.4 DOCUMENTATION OF SAMPLING ACTIVITIES

It is required that all sampling procedures be documented in a sampling plan. Some elements that should be documented in a sampling plan include sampling points, volumes to be drawn, days and times of collection, required equipment, instructions for labelling samples and ensuring chain of custody, and a list of contact persons and telephone numbers in case unexpected difficulties arise during sampling.

2.5 CONDUCTING PROPER ANALYSIS AND QA/QC

In order to demonstrate that a laboratory is producing data of adequate precision, accuracy and sensitivity it is necessary to assess all laboratory procedures at all stages from sampling to reporting. Analytical quality assurance procedures should be based on a system of traceability and feedback. Analytical QA will start with the examination and documentation of all aspects of laboratory management. This will include clearly identifying lines of communication and responsibility, description and documentation of all procedures carried out, and documentation of instrumental and analytical checks. Within this there should be specific control and assessment procedures designed to monitor quantitatively the accuracy and precision of specific assays.

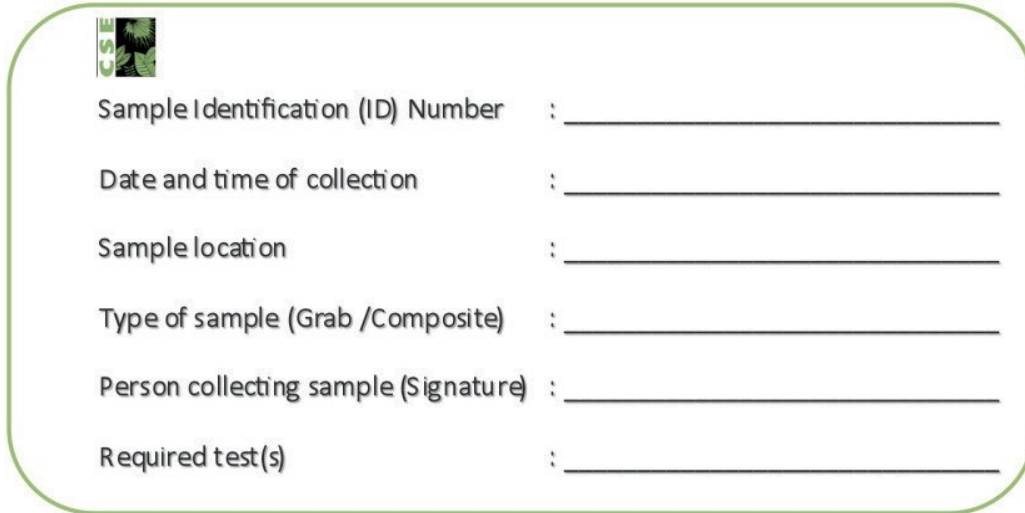
2.6 SAMPLING STAFF AND TRAINING

Staff for collecting samples needs to be adequately trained as handling faecal sludge and wastewater involves health risks from pathogens and other potentially harmful components. In addition, they should be informed about the aim of the sampling and types of sanitation systems. The number of people required for sampling events depends on the sampling method and strategy. Regular health checkups of sampling staff are necessary. Vaccination is required for the sampling staff as well as the analysts working on faecal sludge. Hepatitis A and *Salmonella* vaccines (e.g. Hepatyrix) are a must for sampling staff. A booster is an additional dose of vaccine that can help prolong protective immunity.

2.7 GENERAL SAMPLING PROCEDURE

- ✓ A week to several days prior to the proposed sampling, confirm or schedule sludge processing to ensure that sludge in the appropriate form is available for sampling at the proposed date, time and sampling point.
- ✓ A week to several days prior to the proposed sampling date, schedule/confirm that the contract lab performing the analysis is ready and willing to accept samples on the proposed sampling date.
- ✓ At least one day before collecting samples, assemble the equipment necessary to accomplish the proposed sampling. Ensure that all equipment is clean and in good working order.
- ✓ On the day of sampling, obtain ice for sample storage and transportation and place it in sample coolers.
- ✓ Put on nitrile gloves and other required/desired personal safety measures such as mask, lab coat (full cover apron), boot, goggles etc.
- ✓ Collect the sample using a sampling device. One-day composite sampling takes the first of 24 grab samples from the site to the container. All grab samples should be collected into a 1000 ml Teflon beaker and should be approximately 500 ml in volume. After collecting each grab sample, place the sample in the stainless-steel bucket and record the time of collection. Wait one hour and collect the next grab sample. Repeat the process until all twenty-four grab samples are collected. Between collection of grab samples, the previously collected samples should be kept cool (4–8°C).
- ✓ Once the last grab sample has been collected, thoroughly mix all material accumulated in the stainless steel bucket using a stainless steel rod. After the material is completely mixed, record the current time as the composite sample collection time.
- ✓ For pathogens and indicator organism testing, samples must be collected in sterilized containers. All samples for microbial analyses should be cooled to water-ice temperatures (4–8°C) when collected or very soon thereafter.
- ✓ After mixing, label all sample containers with the minimum information (see *Fig. 1: Label format for sampling*).
- ✓ After labelling, fill each individual sample container with portions of the homogenized sample within the stainless-steel bucket/plastic bucket.
- ✓ After each sample container is filled, seal it with a signed custody seal and place on ice in a cooler for transportation to the laboratory.
- ✓ Prior to delivering the samples to the lab, complete a chain-of-custody sheet to document proper sample handling.
- ✓ After sample delivery, clean all transport equipment according to established procedures and store in a clean, dry area.

Figure 1: Label format for sampling



CSE

Sample Identification (ID) Number : _____

Date and time of collection : _____

Sample location : _____

Type of sample (Grab /Composite) : _____

Person collecting sample (Signature) : _____

Required test(s) : _____

2.8 SAMPLING METHODS FOR FSTP PERFORMANCE EVALUATION

Plastic buckets and ladles were used to collect composite samples from sludge tankers. Wastewater was collected from a specific depth of a chamber by using a cylindrical stainless-steel wastewater sampler attached to a rope. For precise measurement of pH, a portable pH meter was also carried to the field. Personal protective equipment (PPE) like overalls, gloves, face masks, protective eyewear, gumboots and hand sanitizer were taken in the sampling kit. Sampling bottles were properly cleaned, sterilized and attached with appropriate labelling stickers. Sampling location with date and time were clearly stated on each bottle after sampling. Samples were finally transported in iceboxes with leak-proof frozen ice-gel packs.

For the performance evaluation of an FSTP, at least three bottles of samples were collected from each location as described below:

- i. Fresh faecal sludge from a tanker or at the inlet of the drying beds or settler—500 ml (faecal sludge)
- ii. Influent stream entering into the treatment module (wastewater after solid-liquid separation)—1,000 ml (inlet)
- iii. Final effluent from the treatment system—1,500–2,000 ml (outlet)

Composite sampling from the tanker was initiated by assessing the unloading time of septage from the tanker. The slurry removed from the containment was assumed to be stratified into distinct portions within the tanker.

If 'T' is the unloading time and 't' is the time interval between two successive sample collections for the 'n' number of samples of equal volume, $t = T/(n-1)$. Usually 'T' is taken as '10 minutes' 't' = 2.5 minutes and 'n' = 5. The periodicity of sample collection is as follows:

- '0' minutes (at the beginning)
- '0 + t' minutes (2.5 minutes)
- '0 + 2t' minutes: (5 minutes)
- '0 + 3t' minutes: (7.5 minutes)
- '0 + 4t' minutes: (10 minutes, at the end)

A composite sample was prepared by mixing equal volumes of the above five samples in a bucket and then a 500ml sludge sample was collected in a clean 500ml plastic bottle. In all circumstances, the sample bottle was first washed with a representative sample twice or thrice, followed by sampling with minimum head space.

2.9 SAFETY PROCEDURE DURING SAMPLING

To ensure safety and health while sampling, it is important to avoid direct contact between sludge and skin. The following procedures need to be followed for this:

- Wear protective sampling clothes (pants, jacket and boots) that are only worn at the sampling site; store clean clothes and shoes in a closed plastic bag in a clean environment.
- Wear protective eyewear.
- Always wear two pairs of gloves (laboratory gloves covered by long rubber gloves).
- Once all samples are taken and on-site parameters analysed, clean all materials and devices with water directly on the sampling site, taking care not to spread faecal sludge in the environment.
- Once all materials and devices are properly cleaned, and all containers are in the cold box, take off the long rubber gloves.
- Take off your sampling shoes and your sampling clothes, put all sampling clothes and safety gear in a closed bag.
- Take off the laboratory gloves.
- Wash the sampling area with clean water and make sure to leave a clean environment around the sampling site before leaving.

2.10 SAMPLE TRANSPORTATION

Awareness of the properties of FS is necessary in order to understand the challenges faced in its collection and transport. FS properties are primarily influenced by water content, sludge age, and presence of non-biodegradable material and organic material. The containers must have adequate wall thickness to withstand handling during sample collection and transport to the laboratory. Containers with wide mouths are preferred to facilitate transfer of samples from samplers to containers. When samples are collected, they should be placed in clean bottles that are filled completely to the top and stored in an insulated box with cooled gel packs/ice during transportation to the laboratory. Conductivity, temperature and pH should be measured immediately on site, and transport to the laboratory should be as rapid as possible. The maximum transport time to the laboratory is six to twelve hours, and samples should be processed within two hours of receipt at the laboratory.

Chapter 3

3.0 Laboratory organization

Sample preservation and preparation will depend on the parameters to be analysed and available time. In order to ensure reliable and accurate results, the following aspects need to be planned in advance:

- Develop quality assurance and quality control procedures (QA/QC), including blanks, duplicates and calibration;
- Determine parameters and number of analysis;
- Determine required materials and laboratory equipment;
- Determine consumables, including laboratory supplies, chemical reagents and preservation methods;
- Outline laboratory safety measures for analysis;
- Outline the time and logistics required to perform all analyses;
- Calibration of all laboratory instruments before and during use, including standards, dilution curves and blanks;
- Duplicate analysis every samples for all parameters to ensure reproducibility of analytical methods (i.e. larger sample volumes need to be taken so that it is possible for each parameter to be analysed twice and results compared);
- It is recommended that the relative error does not exceed 10 per cent. If the difference between duplicate analyses performed on the same sample is greater than 10 per cent, troubleshooting is required to determine the source of error in the analytical method; and
- Develop standard operating procedures (SOP) for all instruments and ensure SOP training for all lab scientists.

3.1 QUALITY ASSURANCE AND QUALITY CONTROL

Quality control (QC) refers to steps taken to monitor and ensure precision and accuracy of test results. Quality control practices include analysis of quality control samples with each set of samples under analysis. They include calibration standards, certified reference materials, spiked samples, duplicate sample analysis and blanks. Additional QC measures include analysis of blind duplicate samples and participation in various proficiency testing programmes. Quality assurance (QA) refers to completely separate and independent monitoring of laboratory studies and quality control activities. Quality assurance activities include the internal audit programme, review of data packages, evaluation of non-conformances and an annual management review of the quality of data. The principles of good

laboratory practices (GLP) should be implemented in the laboratory to ensure safety, quality and reliability of the data generated.

3.2 ANALYTICAL PARAMETERS

Analytical parameters are determined based on the objectives of the characterization and performance evaluation study. Physical, chemical and biological parameters all influence the design and ongoing operation of treatment plants. The major parameters that should be considered for the characterization of FS include solids concentration, chemical oxygen demand (COD), biochemical oxygen demand (BOD), nutrients, pathogens, and heavy metals. These parameters are the same as those considered for domestic wastewater analysis, but it needs to be emphasized that the characteristics of domestic wastewater and FS are very different.

3.2.1 On-site measurements

It is important to take on-site measures rapidly on-site upon sampling.

Temperature is an important parameter in understanding and predicting rates of biological activity, treatment processes and pathogen die-off. Temperature is measured with a probe immediately after sampling.

pH (potential hydrogen) is a measure of the acidity (< 7) or alkalinity (> 7) of sludge based on the chemical activity of hydrogen ions in solution. pH also has a strong influence on biological processes, including pathogen inactivation. The pH is measured with a probe immediately at the sampling point.

EC (electrical conductivity) is one way to measure inorganic materials, including calcium, bicarbonate, nitrogen, phosphorus, iron, sulphur and other ions, dissolved in a waterbody. It is measured by placing a conductivity probe in the sample and measuring the flow of electricity between the electrodes.

3.2.2 Solid and organic content

Solid and organic content analyses are conducted in the laboratory.

Total solids concentration of FS comes from a variety of organic (volatile) and inorganic (fixed) matter, and comprises floating material, settleable matter, colloidal material, and matter in solution. Parameters that are typically measured include total solids, fractions of volatile or fixed solids, and settleable, suspended or dissolved solids.

Biochemical oxygen demand and chemical oxygen demand of FS are important parameters to monitor as the discharge of FS into the environment can deplete or decrease the oxygen content of waterbodies resulting in the possible death and depletion of aquatic fauna. The oxygen demand is reduced through stabilization and can be achieved by aerobic or anaerobic treatment.

3.2.3 Nutrients content

Nutrients content analyses are conducted in the laboratory.

Total nitrogen (TN) is the sum of nitrate-nitrogen ($\text{NO}_3\text{-N}$), nitrite-nitrogen ($\text{NO}_2\text{-N}$), ammonia-nitrogen ($\text{NH}_3\text{-N}$) and organically bonded nitrogen and each form is generally analysed as a separate component. Total nitrogen is an essential nutrient for plants, animals and microorganisms.

Total nitrogen is quantified as the sum of total Kjeldahl nitrogen (ammonia, organic and reduced nitrogen), NO_3 and NO_2 . NO_3 and NO_2 are expected to be at very low concentrations in anaerobic conditions of septic tanks. Results are reported as mg/L or g/L of total N.

Ammoniacal nitrogen is a measure of the bio-available form of N in anaerobic sludge and is important for growth, but high concentrations can be toxic. It is quantified as nitrogen in ammonium (N-NH_4^+) after a reaction in alkaline solution by titrimetric method.

Total phosphorus (TP) is a measure of the sum of all P (dissolved form—orthophosphate, inorganic and organic). Like nitrogen, phosphorus is an important source of nutrients. It is quantified by the spectrophotometric method after acid digestion.

Heavy metals (iron [Fe], zinc [Zn], nickel [Ni] and lead [Pb]) are important for microbial growth at low concentrations but can inhibit biological processes at high concentrations. They are quantified after a first step of digestion in acid that allows the release of all metals into measurable form.

3.2.4 Pathogenic organisms

Exposure to untreated FS should always be considered as a pathogenic health risk. Adequate reductions in pathogens need to be determined based on the intended end use or disposal option for treated sludge and liquid effluents.

Coliform bacteria

Coliform bacteria are bacteria that populate the intestinal tract and are pervasive in faeces. Their presence in the environment/sample is therefore used as an indicator of faecal contamination. *Escherichia coli* (*E. coli*) is the target organism that has traditionally been used to identify faecal contamination in the environment.

Salmonella

Salmonella spp. are rod-shaped, highly-motile, gram-negative, non-spore-forming, facultatively anaerobic bacteria. This group consists of a range of very closely related bacteria that belongs to the genus *Salmonella* and the family Enterobacteriaceae. *Salmonella* spp. are frequently found in sewage. They are also the most prevalent bacterial pathogens of public health concern. Enteric fever is a collective term given to invasive infections caused by *S. typhi* and *S. paratyphi* that cause typhoid and paratyphoid fever respectively. *S. typhi* is a pathogen that only uses humans as its natural host. Several other *Salmonella* serotypes cause salmonellosis, a food-borne diarrhoeal disease.

Helminths

Helminths are most commonly used as an indicator of the effectiveness of pathogen reduction while analysing FS due to their prevalence in low- and middle-income countries and their persistence following treatment. Helminths (parasitic worms) are eukaryotic parasites, prevalent in about one-third of the world's population. They include nematodes (roundworms), cestodes (flatworms) and trematodes (flukes). The eggs of these parasites are robust and persistent if present in the incoming faecal waste (sludge or sewage) from one infected person and can infect hundreds. *Ascaris lumbricoides*, a type of roundworm, is the most commonly used indicator for testing the performance of a treatment technology as its eggs are most resistant to inactivation and can be identified relatively easily.

3.3 LABORATORY TIMING

A laboratory study plan needs to be developed to optimize time during sampling, transportation, preservation and analysis (including availability of machines). It is essential to analyse samples in a timely fashion to ensure accurate results and prevent changes due to factors such as degradation or volatilization. When several people are involved in sampling and analysing samples, it is useful to have a table in the lab where all tasks are recorded, with the responsible person for each task and their contacts.

A standard operating procedure (SOP) should also include a log book with all analytical activities, responsible parties, use of machines and calibrations,

including dates and comments. Each person working in the laboratory also needs to maintain their own laboratory log book to record all analyses, samples, experimental procedures, QA/QC, information on problems or uncertainties during the analyses, etc. Lab books need to be written in pen, should not have removable pages, and should be kept in the laboratory at all times.

3.4 LABORATORY SAFETY

In order to work in safe conditions, direct contact is to be avoided with sludge and reagents. Sludge should be assumed to contain pathogens such as virus, bacteria, protozoa, parasite and some hazardous chemicals for human health might be used during analysis (e.g. acids). Therefore, the following minimum precautions are to be strictly adhered to:

- Read and follow safety-related indications on the chemical boxes and bottles prior to use.
- Wear a laboratory jacket, closed shoes, and long wears.
- Wear laboratory glasses when manipulating sludge and chemicals.
- Wear vinyl laboratory gloves when manipulating sludge and chemicals.
- Wear an active carbon mask when manipulating sludge and chemicals.
- If you get your skin in contact with chemicals, clean it with detergent and rinse thoroughly with water.
- Always clean the working space and your hand with detergent and ethanol at the end of the analysis.
- Ensure that chemicals and products are stored in a closed and safe location.
- Never eat, drink or smoke in the laboratory; never bring food or drink into the laboratory.
- Keep a first-aid box with essential medicines in the lab.

Chapter 4

4.0 Preparation and preservation of samples

4.1 COMPOSITE PREPARATION

Once the grab samples have been combined, the resulting mixture needs to be homogenized and sub-samples prepared in volumes relevant to the selected analytical procedures. In order to ensure the representativeness of the sub-samples, be sure to include equal and representative quantities of liquid and solids in all jars. Leave approximately 2 cm of headspace at the top of the jar. All containers are to be labelled with the date and sample code and stored at 4°C in the fridge.

4.2 PRESERVATION PROCEDURE

Preservation refers to sample handling processes aimed at preventing or minimizing chemical or biological activity within the sample after it has been collected. Preservation techniques are different for sludge samples and liquid samples or equipment blank samples. Sludge samples are generally preserved by cooling and maintaining samples at 4°C. Depending on the analytical method, liquid samples may be preserved with the combination of a chemical preservative and chilling to 4°C.

To preserve field and laboratory bio-solids samples, cooling to 4°C is (in most cases) the most appropriate method since high-solids sewage sludge cannot be mixed with other preservatives. In addition, laboratory personnel must be notified if chemical preservation is to be done.

4.3 SAMPLE HOLDING TIMES

For all environmental samples, the term 'holding time' refers to the maximum time that can elapse before a sample is analysed and valid results can still be obtained. Table 1 provides general examples of preservation temperatures and maximum holding times, from field collection to analysis, for a typical biosolids sample.

Table 1: Preservation and hold times for analysis of samples

S. no.	Parameters	Preservation	Maximum hold time from field collection to analysis	Type of sample
1.	pH	Store at 4°C	Must be immediately at collection site	Faecal sludge, wastewater, biosolid
2.	Chemical Oxygen Demand (COD)	Store at 4°C	7 days	Faecal sludge, wastewater
3.	Biochemical Oxygen Demand (BOD)	Store at 4°C	6 Hours or when reach at lab	Faecal sludge, wastewater
4.	Total solids (TS)	Store at 4°C	7 days	Faecal sludge, wastewater
5.	Total dissolved solids (TDS)	Store at 4°C	7 days	Faecal sludge, wastewater
6.	Total suspended solids (TSS)	Store at 4°C	7 days	Faecal sludge, wastewater
7.	Volatile solids (VS)	Store at 4°C	7 days	Faecal sludge, wastewater
8.	Ammoniacal nitrogen (AN)	Store at 4°C	28 days	Faecal sludge, wastewater
9.	Total Kjeldahl nitrogen (TKN)	Store at 4°C	28 days	Faecal sludge, wastewater
10.	Nitrate (NO ₃ -)	Store at 4°C	7 days	Faecal sludge, wastewater
11.	Nitrite (NO ₂ -)	Store at 4°C	7 days	Faecal sludge, wastewater
12.	Total phosphorus (TP)	Store at 4°C	28 days	Faecal sludge, wastewater
13.	Electrical conductivity (EC)	Store at 4°C	28 days	Biosolid
14.	Moisture	Store at 4°C	28 days	Biosolid
15.	Total organic carbon (TOC)	Store at 4°C	28 days	Biosolid
16.	Total nitrogen (TN)	Store at 4°C	14 days	Faecal sludge, wastewater, biosolid
17.	Faecal coliform (FC)	Store at 4°C	24 hours	Faecal sludge, wastewater, biosolid
18.	<i>E. coli</i>	Store at 4°C	24 hours	Faecal sludge, wastewater, biosolid
19.	<i>Salmonella</i>	Store at 4°C	24 hours	Biosolid
20.	Helminth ova	Store at 4-10°C	7 days	Faecal sludge, biosolid
21.	Potassium	Store at 4°C	6 months	Biosolid
22.	Arsenic	Store at 4°C	6 months	Biosolid
23.	Cadmium	Store at 4°C	6 months	Biosolid
24.	Chromium	Store at 4°C	6 months	Biosolid
25.	Copper	Store at 4°C	6 months	Biosolid
26.	Lead	Store at 4°C	6 months	Biosolid
27.	Mercury	Store at 4°C	28 days	Biosolid
28.	Nickel	Store at 4°C	6 months	Biosolid
29.	Zinc	Store at 4°C	6 months	Biosolid

Chapter 5

5.0 Analysis protocols of wastewater and faecal sludge

5.1 STANDARD OPERATING PROCEDURE FOR pH DETERMINATION

Scope and field of application

This method is an electrochemical procedure for measuring pH in water, wastewater or faecal sludge samples.

Interferences

Samples with very low or very high pH may give incorrect readings on the meter. For samples with a true pH of >10 , the measured pH may be incorrectly low. This error can be minimized by using a low-sodium-error electrode. Strong acid solutions, with a true pH of <1 , may give incorrectly high pH measurements. Errors will occur when the electrodes become coated. If an electrode becomes coated with an oily material that will not rinse free, the electrode can be cleaned with an ultrasonic bath or be washed with detergent, rinsed several times with water, placed in 1:10 HCl so that the lower third of the electrode is submerged, and then thoroughly rinsed with water. It can also be cleaned as per the manufacturer's instructions.

Safety precautions

- Always use safety goggles, gloves and laboratory coat while working in laboratory.
- After the analysis clean bottles and beakers with clear water and keep them for drying.
- Dispose of the used gloves after completion of analysis.
- Clean hands using antiseptic soap.
- Avoid chemical/sample spillage and contact with skin.
- Disinfect hands after washing with soap.

Apparatus

- pH meter with means for temperature compensation
- Glass electrode

- Reference electrode—a silver chloride or other reference electrode of constant potential may be used
- 50 ml beaker
- Thermometer and/or temperature sensor for automatic compensation
- Analytical balance capable of weighing 0.1 g

Procedure

Sample preparation

Faecal sludge samples are mostly heterogeneous and concentrated that needs to be liquefied by adding water before pH estimation. To 20 g of FS sample in a 50 ml beaker, add 20 ml of distilled water (1:1), cover, and continuously stir the suspension for 5 minutes. Additional dilutions are allowed if working with hygroscopic wastes and salts or other problematic matrices. Let the waste suspension stand for about 15 minutes to allow most of the suspended waste to settle out from the suspension or filter or centrifuge off the aqueous phase for pH measurement. Report the dilution with the result.

However, samples that are already in liquid form can be analysed directly without any further addition of water.

Note 1: If the waste is hygroscopic and absorbs all the reagent water, begin the experiment again using 20 g of waste and 40 ml of reagent water.

Note 2: If the supernatant is multiphasic, decant the oily phase and measure the pH of the aqueous phase. The electrode may need to be cleaned if it becomes coated with an oily material.

Note 3: If samples are already in liquid form and give stable readings, they can be measured directly without any dilution.

Measurement of pH

Adjust the electrodes in the clamps of the electrode holder so that upon lowering the electrodes into the beaker the glass electrode will be immersed just deep enough into the clear supernatant to establish good electrical contact through the ground glass joint or the fibre-capillary hole. Insert the electrode into the sample solution in this manner.

For combination electrodes, immerse just below the suspension. If the sample temperature differs by more than 2°C from the buffer solution, the measured pH values must be corrected.

Results

Report the results as 'sample pH measured at __°C' where ' __°C' is the temperature at which the test was conducted.

5.2 STANDARD OPERATING PROCEDURE FOR TOTAL SOLIDS

Scope and field of application

Total solids (TS) are determined in a wide variety of liquid and semi-liquid materials. These include potable waters, domestic and industrial waters, polluted waters and sludge produced from treatment processes. It is of particular importance for the efficient operation of a treatment plant.

Principle

An appropriate volume of well-mixed sample is evaporated to dryness contained in a weighed evaporating dish in a hot air oven at 103-105°C, the residue remaining after drying is cooled and weighed. The residual material in the evaporating dish is classified as total solids, and may consist of organic, inorganic, dissolved, suspended or volatile matter.

Interferences

Highly mineralized water with a significant concentration of calcium, magnesium, chloride and sulphate may be hygroscopic and require prolonged drying, proper desiccation and rapid weighing. Exclude large floating particles from the sample if it is determined that their inclusion is not desired in the final result.

Safety precautions

- Always use safety goggles, gloves and laboratory coat.
- Wear suitable protective gloves (heat resistant) when taking out evaporating dish from the oven.
- Clean all the dishes (crucibles), glassware and bottles well when analysis is completed
- Clean hands with antiseptic soap and disinfectant.

Apparatus

- Evaporating dishes: dishes of 50–100 ml
- Drying oven for operation at 103–105°C
- Water bath
- Desiccator (provided with a desiccant containing a colour indicator of moisture concentration)
- Analytical balance (capable of weighing to 0.1 mg)
- Magnetic stirrer and bar
- Wide-bore pipettes
- Forceps
- Beaker

Procedure

Sample analysis

- Choose a sample volume that will yield a residue between 2.5 and 200 mg.
- Stir the sample with a magnetic stirrer for homogeneous samples, pipette out a measured volume of well-mixed sample from the approximate midpoint of the container.
- If sample contains solids in the range up to 20,000 mg/L, transfer carefully 20–50 ml of well-mixed sample into an evaporating dish. If sample contains solids more than 20,000 mg/l, transfer carefully 10–20 g of well-mixed sample into evaporating dish and weigh.
- Dry evaporated sample for at least 1 hour in an oven at 103–105°C, cool the dish in the desiccator to balance temperature, and weigh.
- Weigh until a constant weight is obtained.

$$\text{Total solids} \frac{\text{mg}}{\text{L}} = (A - B) \times \frac{1000}{\text{Sample volume (ml)}}$$

where

A =	Weight of dried residue + dish (mg)
B =	Weight of dish (mg)
C =	Weight of wet sample + dish (mg)

5.3 STANDARD OPERATING PROCEDURE FOR TOTAL SUSPENDED SOLIDS

Scope and field of application

Total suspended solids (TSS) are useful determinants in the analysis of polluted, reused and wastewaters. They are used to evaluate the strength of domestic/ industrial wastewaters and determine the efficiency of treatment units, such as settling tanks, biological filters, and the activated sludge.

Principle

A measured volume of well-mixed sample is vacuum filtered through a dried pre-weighed glass fibre filter. The filters and residue are dried to a constant weight at 103–105°C. The increase in weight of the filter represents the total suspended solids.

Interferences

- Exclude isolated large floating particles.
- Samples high in dissolved solids must be washed adequately.
- Loss in mass of the rinsed glass fibre filters must be taken into the final calculation.
- The larger the sample, the smaller the factor applied in the calculation, but avoid prolonged filtrations.

Safety precautions

- Always use safety goggles, gloves and laboratory coat.
- Wear suitable protective gloves when removing evaporating dish from the oven.
- Extreme care must be taken when using glassware, vacuum pumps and ovens.
- Cleaning all glassware and bottles well when analysis is completed
- Clean the hands with antiseptic soap and disinfectant.

Apparatus

- Evaporating dishes: dishes of 50–100 ml
- Drying oven for operation at 103–105°C
- Filtration apparatus
- Vacuum pump
- Whatman glass fibre filter or cellulose filters
- Desiccator (provided with a desiccant containing a colour indicator of moisture concentration)
- Analytical balance (capable of weighing to 0.1 mg)

- Magnetic stirrer and bar
- Wide-bore pipettes
- Forceps
- Beaker

Procedure

- Prepare glass fibre filter paper and evaporating dish
- Use Whatman glass fibre filter paper and mark the filter paper.
- Place filter paper on the evaporating dish as a support.
- Filter paper is heated to 103–105°C for 1 hour. Store and cool filter paper in desiccator until needed. Weigh immediately before use.

Sample analysis

- Choose a sample volume that will yield a residue between 2.5 and 200 mg.
- Stir sample with a magnetic stirrer to homogeneous samples, pipette a measured volume of well-mixed sample from the approximate midpoint of the container but not in the vortex.
- Insert filter paper (GF/C paper) with wrinkled side up into filtration apparatus connected vacuum pump and spray few distilled water into filter paper and suction.
- Transfer carefully well-mixed sample 20–50 ml into filter paper on filtration apparatus and continue suction until the filtration is complete.
- When complete filtration takes more than 10 minutes, increase filter diameter or decrease sample volume.
- Wash filter with three successive 10 ml volumes of reagent-grade water, allowing complete drainage between washings, and continue suction for about 3 minutes after filtration is complete. (Samples with high dissolved solids may require additional washings.)
- Carefully remove filter paper from filtration apparatus and transfer to an evaporating dish as a support.
- Dry sample for at least 1 hour in an oven at 103–105°C, cool dish in a desiccator to balance the temperature and weigh.
- Weigh until a constant weight is obtained.

$$\begin{aligned} \text{Total suspended solids } \left(\frac{\text{mg}}{\text{L}} \right) \\ = (A - B) \times \frac{1000}{\text{sample volume (ml)}} \end{aligned}$$

where A = Weight of filter + dried residue (mg)
 B = Weight of filter (mg)

5.4 STANDARD OPERATING PROCEDURE FOR TOTAL DISSOLVED SOLIDS (TDS)

Scope and field of application

Dissolved solids are useful determinants in the analysis of polluted, reused and wastewaters. They are used to evaluate wastewater that contains a high fraction of colloidal solids. The size of colloidal particles in wastewater is typically in the range of 0.01 to 1.0 μm .

Principle

A well-mixed sample is filtered through a standard glass fibre filter, and the filtrate is evaporated to dryness in a weighed dish and dried to a constant weight at 180°C. The increase in dish weight represents the total dissolved solids. This procedure may be used for drying at other temperatures.

Interferences

Highly mineralized waters with a considerable calcium, magnesium, chloride, and/or sulphate content may be hygroscopic and require prolonged drying, proper desiccation and rapid weighing.

Samples high in bicarbonate require careful and possibly prolonged drying at 180°C to insure complete conversion of bicarbonate to carbonate. Because excessive residue in the dish may form a water-trapping crust, limit sample to no more than 200 mg residue.

Safety precautions

- Always use safety goggles, gloves and laboratory coat.
- Wear gloves suitable to prevent high temperatures when removing evaporating dish from the oven.
- Exercise care when using glassware, vacuum pumps and ovens.
- Cleaning all glassware and bottles when finished analysis.
- Clean the hands with antiseptic soap and disinfectant.

Apparatus

- Evaporating dishes: dishes of 50–100 ml
- Drying oven for operation at 103–105°C
- Filtration apparatus
- Vacuum pump
- Water bath
- Whatman glass fibre filter

- Desiccator (provided with a desiccant containing a color indicator of moisture concentration)
- Analytical balance (capable of weighing to 0.1 mg)
- Magnetic stirrer
- Magnetic bar
- Wide-bore pipettes
- Forceps
- Beaker

Procedure

- Prepare the glass fibre filter paper and evaporating dish
- Dry the dish to 103–105°C for 1 hour. Store and cool the dish in desiccator until needed. Weigh the dish before placing the sample.

Sample analysis

- Choose a sample volume that will yield a residue between 2.5 and 200 mg.
- Stir sample with a magnetic stirrer to make homogeneous sample, pipette a measured volume of well-mixed sample from the approximate midpoint of the container but not in the vortex.
- Insert filter paper (GF/C paper) with the wrinkled side up into filtration apparatus that connects vacuum pump and spray some distilled water into the filter paper with suction.
- Transfer carefully 20–50 ml of the well-mixed sample into the filter paper on the filtration apparatus and continue suction.
- Wash filter with three successive 10-ml volumes of reagent-grade water, allowing complete drainage between washings, and continue suction for about 3 minutes after filtration is complete.
- Transfer total filtrate sample (with wash water) to a weighed evaporating dish and evaporate to dryness on a water bath.
- Dry evaporated sample for at least 1 hour in an oven at 180°C; cool dish in desiccator to balance the temperature and weigh.
- Weigh until a constant weight is obtained.

$$\text{Total dissolved solids (mg/L)} = \frac{(A - B) * 1000}{\text{Sample Volume (ml)}}$$

where A = Weight of dried residue + dish (mg) B = Weight of dish (mg)

5.5 STANDARD OPERATING PROCEDURE FOR TOTAL FIXED SOLIDS (TFS) AND TOTAL VOLATILE SOLIDS (TVS)

Principle

The residue from the dried the sample at 103–105°C mentioned in the method for TS estimation is ignited to constant weight at 550°C. The remaining solids represent the fixed total, dissolved or suspended solids while the weight lost on ignition is the volatile solids. The determination is useful in control of wastewater treatment plant operation because it offers a rough estimate of the amount of organic matter present in the solid fraction of wastewater, activated sludge and industrial wastes.

Interferences

Negative errors in the volatile solids may be produced by loss of volatile matter during drying. Determination of low concentrations of volatile solids in the presence of high fixed solids concentrations may be subject to considerable error.

Safety precautions

- Always use safety goggles, gloves and laboratory coat.
- Wear gloves and use long clamp suitable to prevent high temperatures when removing evaporating dish from the oven and furnace.
- Exercise care when using glassware, vacuum pumps and ovens.
- Good cleaning all glassware and bottles when finished analysis.
- Clean the hands with antiseptic soap and disinfect.

Apparatus

- Evaporating dishes: dishes of 50–100 ml
- Muffle furnace for operation at 550°C
- Oven for operation at 103–105°C
- Water bath
- Desiccator (provided with a desiccant containing a color indicator of moisture concentration)
- Analytical balance (capable of weighing to 0.1 mg)
- Vacuum pump
- Magnetic stirrer
- Magnetic bar
- Wide-bore pipettes
- Forceps
- Beaker

Procedure

Sample analysis

- Choose a sample volume that will yield a residue between 2.5 and 200 mg.
- Stir sample with a magnetic stirrer to homogeneous samples, pipette a measured volume of well-mixed sample from the approximate midpoint of the container but not in the vortex.
- If sample contains solids below 20,000 mg/L, transfer carefully 20–50 ml of well-mixed sample into an evaporating dish. If the solid content in the sample is in the range up to 20,000 mg/l, transfer carefully 10–20 g of well-mixed sample into evaporating dish and weight.
- Evaporate to dryness on a water bath.
- Dry evaporated sample for at least 1 hour in an oven at 103–105°C, cool dish in desiccator to balance the temperature, and weigh.
- Ignite dry sample at 550°C for 15–20 minutes.
- Weigh dish as soon as it has cooled to room temperature. Weigh until a constant weight is obtained.

Calculation

$$\text{Total volatile solids (mg/L)} = \frac{(A-B) \times 1000}{\text{Sample Volume (ml)}}$$

$$\% \text{ total volatile solids} = \frac{(A-B) \times 100}{(A-C)}$$

$$\text{Total fixed solids (mg/L)} = \frac{(B-C) \times 1000}{\text{Sample Volume (ml)}}$$

$$\% \text{ total fixed solids} = \frac{(B-C) \times 100}{(A-C)}$$

where A = Weight of dried residue + dish before ignition (mg)
 B = Weight of dried residue + dish after ignition (mg)
 C = Weight of dish (mg)

5.6 STANDARD OPERATING PROCEDURE FOR VOLATILE SUSPENDED SOLIDS (VSS)

Principle

The residue from the TS determination method is ignited to a constant weight at 550°C. The remaining solids represent the fixed total, dissolved or suspended solids while the weight lost on ignition is the volatile solids. The determination

is useful in controlling wastewater treatment plant operation because it offers a rough estimate of the amount of organic matter present in the solid fraction of wastewater, activated sludge and industrial wastes.

Interferences

Negative errors in the volatile solids may be produced by loss of volatile matter during drying.

Determination of low concentrations of volatile solids in the presence of high fixed solids concentrations may be subjected to considerable error.

Safety precautions

- Always use safety goggles, gloves and laboratory coat.
- Wear gloves and use long clamp suitable to prevent high temperatures when removing evaporating dish from the oven and furnace.
- Exercise care when using glassware, vacuum pumps and ovens.
- Good cleaning all glassware and bottles when finished analysis.
- Clean the hands with antiseptic soap and disinfect.

Apparatus

- Evaporating dishes: dishes of 50–100 ml
- Muffle furnace for operation at 550°C
- Oven for operation at 103–105°C
- Filtration apparatus
- Whatman glass fibre filter
- Desiccator (provided with a desiccant containing a colour indicator of moisture concentration)
- Analytical balance (capable of weighing to 0.1 mg)
- Vacuum pump
- Magnetic stirrer and bar
- Wide-bore pipettes
- Forceps
- Beaker

Procedure

- Prepare glass fibre filter paper and evaporating dish.
- Use Whatman glass fibre filter paper and mark the filter paper.
- Place filter paper on the evaporating dish as a support.
- Heat filter paper to 103–105°C for one hour. Store and cool filter paper in a desiccator until needed. Weigh immediately before use.

Sample analysis

- Choose a sample volume that will yield a residue between 2.5 and 200 mg.
- Stir sample with a magnetic stirrer to homogeneous samples, pipette a measured volume of well-mixed sample from the approximate midpoint of the container but not in the vortex.
- Insert filter paper (GF/C paper) with wrinkled side up into filtration apparatus that connected vacuum pump and spray some distilled water into the filter paper and suction.
- Transfer carefully 20–50 ml of the well-mixed sample into the filter paper on the filtration apparatus and continue suction.
- When complete filtration takes more than 10 minutes, increase the filter diameter or decrease sample volume.
- Wash filter with three successive 10-ml volumes of reagent-grade water, allowing complete drainage between washings, and continue suction for about 3 minutes after filtration is complete. Note: Samples with high dissolved solids may require additional washings.
- Carefully remove filter paper from filtration apparatus and transfer to an evaporating dish as a support.
- Dry sample for at least 1 h in an oven at 103–105°C, cool dish in a desiccator to balance temperature, and weigh.
- Ignite dry sample at 550°C for 15–20 minutes.
- Weigh until a constant weight is obtained, or until weight change is less than 4 per cent of previous weight or 0.5 mg, whichever is less.

Calculation

$$\text{mg of volatile suspended solids/L} = \frac{(A-B) \times 1000}{\text{Sample volume (ml)}}$$

where A = Weight of dried residue + dish before ignition, mg
 B = Weight of dried residue + dish after ignition, mg

5.7 STANDARD OPERATING PROCEDURE FOR SLUDGE VOLUME INDEX

Principle

Sludge volume index (SVI) is the volume of millilitres occupied by 1 gram of a suspension after 30 minutes settling. SVI typically is used to monitor settling characteristics of activated sludge and other biological suspensions. Although SVI is not supported theoretically, experience has shown it to be useful in routine process control.

Safety precautions

- Always use safety goggles, gloves and laboratory coat.
- Cleaning all glassware and bottles thoroughly when analysis is complete.
- Clean hands with antiseptic soap and disinfectant.

Apparatus

- Imhoff cone or cylinder

Procedure

- Determine the suspended solids concentration of a well-mixed sample of suspension.
- Fill an Imhoff cone or cylinder to the 1-litre mark with a well-mixed sample.
- Settle for 30 minutes.
- Record the volume of sludge at 30 minutes as the settled volume.

Calculation

$$\text{Sludge volume index ml/g} = \frac{\text{Settled sludge volume (ml/L)} \times 1000}{\text{Suspended solids (mg/L)}}$$

Interpretation of SVI results

- SVI: < 100 ml/g: Old sludge, possible pin floc, increasing effluent turbidity
- SVI: 100–250 ml/g: Normal operation, good settling, low effluent turbidity
- SVI: > 250 ml/g: Bulking sludge, poor settling, high effluent turbidity

5.8 STANDARD OPERATING PROCEDURE FOR TESTING OF OIL AND GREASE IN FAECAL SLUDGE BY LIQUID-LIQUID, PARTITION-GRAVIMETRIC METHOD

Scope and application

Oil and grease are defined as any material recovered as a substance soluble in the solvent. It includes other material extracted by the solvent from an acidified sample (such as sulphur compounds, certain organic dyes, and chlorophyll) and not volatilized during the test.

It is important to understand that unlike some constituents that represent distinct chemical elements, ions, compounds or groups of compounds, oils and greases are defined by the method used for their determination. In detailed studies involving wastewaters and solid matrices, it was shown that n-hexane produced results statistically different from results produced by trichlorotrifluoroethane. They also may be suitable for most industrial wastewaters or treated effluents containing these materials, although sample complexity may result in either low or high results because of lack of analytical specificity. The method is not applicable to measurement of low-boiling fractions that volatilize at temperatures below 85°C.

Principle

This method is a liquid-liquid, partition-gravimetric method. Dissolved or emulsified oil and grease is extracted from faecal sludge by intimate contact with an extracting solvent (n-hexane) and estimation is made gravimetrically. Some extractables, especially unsaturated fats and fatty acids, oxidize readily; hence, special precautions regarding temperature and solvent vapour displacement are included to minimize this effect. Organic solvents shaken with some samples may form an emulsion that is very difficult to break. This method includes a means for handling such emulsions. Recovery of solvents is discussed. Solvent recovery can reduce both vapour emissions to the atmosphere and costs.

Sample collection, preservation and storage

Collect a representative grab sample in a wide-mouth glass bottle that has been washed with soap, rinsed with water, and finally rinsed with solvent to remove any residues that might interfere with the analysis. As an alternative to solvent rinsing, cap bottle with aluminium foil and bake at 200–250°C for at least 1 hour. Use PTFE-lined caps for sample bottles; clean liners as above, but limit temperature to 110–200°C. Collect a separate sample for an oil and grease determination. Collect replicate samples for replicate analyses or known-

addition QA checks. Typically, collect wastewater samples of approximately 1 litre. If sample concentration is expected to be greater than 1,000 mg extractable material/L, collect proportionately smaller volumes. If analysis is to be delayed for more than 2 hours, acidify to pH 2 or lower with either 1:1 HCl or 1:1 H₂SO₄ and refrigerate. When information is required about average grease concentration over an extended period, examine individual portions collected at prescribed time intervals to eliminate losses of grease on sampling equipment during collection of a composite sample. In sampling sludges, take every possible precaution to obtain a representative sample. When analysis cannot be made within 2 hours, preserve samples with 1 mL conc. HCl/80 g sample and refrigerate. Never preserve samples with CHCl₃ or sodium benzoate.

Apparatus

- Separatory funnel, 2 L, with Teflon or equivalent (TFE) stopcock;
- Distilling flask, 125 ml;
- Liquid funnel, glass;
- Filter paper, Whatman No. 40 or equivalent, 11-cm diameter;
- Centrifuge, capable of spinning at least four 100-ml glass centrifuge tubes at 2400 rpm or more;
- Centrifuge tubes, 100 ml, glass;
- Water bath, capable of maintaining 85°C;
- Vacuum pump or other source of vacuum;
- Distilling adapter with drip tip/ solvent recovery equipment;
- Ice bath;
- Waste receptacle, for used solvent;
- Desiccator.

Reagents

- Hydrochloric or sulphuric acid, 1:1

Mix equal volumes of acid and reagent water.

- n-Hexane

85 per cent minimum purity, 99 per cent minimum saturated isomers, residue less than 1 mg/L; distil if necessary. Do not use any plastic tubing to transfer solvent between containers.

- Sodium sulphate, Na₂SO₄, anhydrous crystal

Dry at 200–250°C for 24 hours.

Procedure

- When a sample is brought into the laboratory, either mark sample bottle at the water meniscus or weigh the bottle, for later determination of sample volume.
- If sample has not been acidified previously, acidify with either 1:1 HCl or 1:1 H₂SO₄ to pH 2 or lower (generally, 5 mL is sufficient for a 1 L sample).
- Using liquid funnel, transfer sample to a separatory funnel.
- Carefully rinse the sample bottle with 30 mL n-hexane, the extracting solvent and add solvent washings to separatory funnel.
- Shake the separatory funnel with sample and n-hexane vigorously for 2 minutes. However, if it is suspected that a stable emulsion will form, shake gently for 5–10 minutes. Let aqueous and organic (solvent) layers separate.
- Drain aqueous layer and small amount of organic layer into original sample container.
- Drain solvent layer through a funnel containing a filter paper and 10 g Na₂SO₄, both of which have been solvent-rinsed, into a clean, tared distilling flask containing boiling chips.
- If a clear solvent layer cannot be obtained and an emulsion of more than about 5 mL exists, drain emulsion and solvent layers into a glass centrifuge tube and centrifuge for 5 minutes at approximately 2,400 rpm. Transfer centrifuged material to an appropriate separatory funnel and drain solvent layer through a funnel with a filter paper and 10 g Na₂SO₄, both of which have been pre-rinsed, into a clean, tared distilling flask. Recombine aqueous layers and any remaining emulsion or solids in separatory funnel.
- For samples with <5 mL of emulsion, drain only the clear solvent through a funnel with pre-moistened filter paper and 10 g Na₂SO₄. Recombine aqueous layers and any remaining emulsion or solids in separatory funnel.
- Extract twice more with 30 mL solvent each time, but first rinse sample container with each solvent portion.
- Repeat centrifugation step if emulsion persists in subsequent extraction steps. Combine extracts in tared distilling flask, and include in flask a final rinsing of filter and Na₂SO₄ with an additional 10–20 mL solvent.
- Distil solvent from flask in a water bath at 85°C. To maximize solvent recovery, fit distillation flask with a distillation adapter equipped with a drip tip and collect solvent in an ice-bath cooled receiver. When visible solvent condensation stops, replace bent distillation apparatus with vacuum/air adapter connected to vacuum source. Immediately draw air through flask with an applied vacuum for the final 1 minute.
- Remove flask from bath and wipe outside surface to remove moisture. Cool in desiccator until a constant weight is obtained.

-
- To determine initial sample volume, either fill sample bottle to mark with water and then pour water into a 1-L graduated cylinder, or weigh empty container and cap and calculate the sample volume by difference from the initial weight (assuming a sample density of 1.00).

Calculation

Calculate oil and grease in sample as follows:

$$\text{mg oil and grease/L} = W_r/V_s$$

where:

W_r = total weight of flask and residue, minus tare weight of flask, mg, and

V_s = initial sample volume, L

5.9 STANDARD OPERATING PROCEDURE FOR CHEMICAL OXYGEN DEMAND (COD) BY COLORIMETRIC METHOD

Scope and application

- The COD (chemical oxygen demand) expresses the amount of oxygen originating from potassium dichromate that reacts with the oxidizable substances contained in 1 L of water under the working conditions of the specified procedure.
- It is an important and rapidly measured parameter to measure the amount of organic compounds in stream and industrial waste studies, and in operational control of wastewater treatment plants. It is also applicable for measurements on human excreta.

Summary

The water sample is oxidized with a hot sulphuric solution of potassium dichromate, with silver sulphate as a catalyst. Chloride is masked with mercury sulphate. The concentration of unconsumed yellow CrO_4^{2-} ions or, respectively, of green Cr^{3+} ions are then determined photometrically. The method is analogous to EPA 410.4, US Standard Methods 5220 D and ISO 6060

Interferences

Difficulties caused by the presence of chlorides in the sample are overcome by the addition of mercuric sulphate to samples before digesting. The chloride ion is then eliminated from the reaction by forming a soluble mercuric chloride complex.

A catalyst must be used to include some organic compounds (e.g. acetic acid), while other biological compounds (e.g. cellulose), which are not important, are included in the determination. Pyridine is not oxidized even in the presence of the catalyst.

Sampling

- Collect samples preferably in glass bottles.
- Test unstable samples without delay.
- Preserve samples by acidifying with concentrated sulphuric acid to pH 2.
- Determine COD on well-shaken samples. Settled samples may also be analysed if requested.
- Use a 3 ml pipette to measure out samples.

Safety precautions

- Handle reagents with care.
- Always use safety goggles, gloves and laboratory coat while working in laboratory.
- Wear face shield and protect hands from heat produced when contents of the vessels are mixed.
- After the analysis clean bottles and beakers with water and then dry.
- Dispose of the used gloves after completion of analysis.
- Clean hands using antiseptic soap.
- Avoid spillage and contact with skin. In the latter case, use copious quantities of cold water to wash skin and call for medical attention.

Apparatus

- Thermoreactor (Merck Spectroquant TR320)
- Photometer (Merck Spectroquant NOVA 60A)
- Cuvette
- Vortex mixture

Table 2: Reagent details

Measuring range (mg/L COD)	4.0–40.0	10–150	100–1500	500–10000
Solution A	1.14538.0065	1.14538.0065	1.14538.0065	1.14679.0495
Cat. No. Volume	0.30 ml	0.30 ml	0.30 ml	2.20 ml
Solution B	1.14681.0495	1.14682.0495	1.14539.0495	1.14680.0495
Cat. No. Volume	2.85ml	2.85 ml	2.30 ml	0.80 ml

Sample preparation

- Analyse immediately after sampling.
- Homogenize the samples.
- Check the chloride content with the Merck quant[®] Chloride Test. Samples containing more than 2,000 or, respectively, 5,000 mg/l Cl⁻ must be diluted with distilled water prior to determining COD.

Procedure

- Pipette solution A and solution B into an empty cell (free of scratches and organic impurities) according to the desired measuring range and mix (see *Table 2: Reagent details*). Take care not to exceed the stated volumes.
- If the expected range of COD is from 4 to 1500 mg/L, then add 3 ml of sample above mixture. Only 1 ml of sample is required if the expected range of COD is from 500 to 10000 mg/L.
- Suspend any bottom sediment present in the cell by swirling.
- Tightly attach the screw cap to the cell.
- In all subsequent steps the cell must be held only by the screw cap.
- Vigorously mix the contents of the reaction cell.
- Heat the reaction cell for 120 minutes at 148°C in the preheated thermoreactor. Remove the hot reaction cell from the thermoreactor and allow to cool in the cell rack. Do not cool with cold water.
- Wait 10 minutes, shake the cell and return to the rack for complete cooling to room temperature (cooling time at least 30 minutes).
- Select the respective programme in the spectroquant photometer.
- Measure the sample in the photometer. The results are displayed as mg/L

Result interpretation

The concentration of nitrate in mg/L can be directly read from the spectroquant photometer. If the sample is diluted before or during analysis, then the dilution factor has to be multiplied with the photometer reading

5.10 STANDARD OPERATING PROCEDURE FOR AMMONIACAL NITROGEN

Scope and application

- Ammonia is naturally being present in surface and wastewaters.
- All these forms of nitrogen as well as nitrogen gas are biochemically interconvertible and are components of the nitrogen cycle.
- The method covers the range from about 10–25 mg/L for titrimetric procedure.

Summary

- The sample is buffered at pH 9.5 with borate buffer to decrease hydrolysis of cyanates and organic nitrogen compounds.
- It is distilled into a solution of boric acid when titration is to be used.
- The ammonia in the distillate is determined titrimetrically with standard sulphuric acid and a mixed indicator together with a pH meter.

Interferences

Glycine, urea, glutamic acid, cyanates and acetamide hydrolyze very slowly in solution on standing but of these only urea and cyanates hydrolyze on distillation at pH 9.5.

Sampling

- Most reliable results are obtained on fresh samples.
- Destroy residual chlorine immediately (by adding chemicals such as sodium sulphite, sodium bisulphite or sodium metabisulphite) after sample collection to prevent its reaction with ammonia.
- If an immediate analysis is not possible, preserve samples by acidifying to pH of 1.5–2.0 with 0.8 ml conc. H_2SO_4/L and store at 4°C.
- If acid preservation is used, neutralize samples with NaOH or KOH immediately before making the determination.

Safety precautions

- Always use safety goggles, gloves and laboratory coat while working in laboratory.
- Use eye and hand protection when preparing acid or handling color reagent.
- Prepare and keep colour reagent in fume hood.

Apparatus

- VELP distillation unit
- pH meter

Reagents

- Ammonia-free water

Eliminate traces of ammonia in distilled water by adding 0.1ml sulphuric acid to 1 L distilled water and redistill. Alternatively treat distilled water with enough bromine or chlorine water to produce a free halogen residue of 2–5 mg/L and redistill after standing for 1 hour.

-
- 0.1N NaOH

Dissolve 4 g NaOH in 1L distilled water.

- 1N NaOH

Dissolve 40 g NaOH in 1 L ammonia-free distilled water.

- Borate buffer solution

Add 88 mL of 0.1N NaOH solution to 500 ml of 0.025M di-sodium tetra borate-hydrous ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) solution (9.5 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ hydrous per litre) or (5.0 g $\text{Na}_2\text{B}_4\text{O}_7$ anhydrous per liter) and dilute to 1L

- Mixed indicator solution

Dissolve 200 mg methyl red indicator in 100 ml 95 per cent ethyl or isopropyl alcohol or ethanol. Dissolve 100 mg methylene blue in 50 ml 95 per cent ethyl or isopropyl alcohol or ethanol. Combine solutions. Prepare monthly.

- Boric acid solution

Dissolve 20 g H_3BO_3 in ammonia-free distilled water, and dilute to 1 L. Prepare monthly.

Standard sulphuric acid titrant (0.02 N)

Dissolve 0.5 ml concentrated sulphuric acid in distilled water and dilute to 1 L. Weigh out about 1.325 g anhydrous sodium carbonate, previously dried at 270°C. Dissolve in distilled water and make up to 250 ml in a volumetric flask–this is 0.1 N.

Note: Do not keep longer than 1 week. Titrate the sulphuric acid solution against 25 ml of sodium carbonate solution using bromocresol green-methyl red mixed indicator. Calculate the normality of the sulphuric acid.

Normality of H_2SO_4 solution = 25 x 0.1/Vol. of H_2SO_4 used

Procedure

- Preparation of equipment: Keep 500 ml ammonia-free water in a bottle connected to the steam distillation apparatus and 50 ml of boric acid with 5 drops of mixed indicator.
- Add a few glass beads and use this mixture to steam out the distillation apparatus until distillate shows no traces of ammonia.
- Add appropriate amount (see *Table 3: Sample volume selection table*) of sample to distillation flask.
- Add 20 ml borate buffer to the sample in the distillation flask and adjust pH to 9.5 with 6 N NaOH solution.
- Take 50 ml of boric acid in the conical flask and add 5 drops of mixed indicator which turns the colour of the solution to purple.
- Distill for 5 min and collect nearly 100 ml distillate (containing ammonia) into the 50 ml boric acid solution which turns the solution into green colour.
- Titrate the distillate with standard 0.02N sulphuric acid; titrate until green turns purple.
- Carry a blank through all steps of the procedure and apply the necessary correction to the results.

Table 3: Sample volume selection table

Ammonia nitrogen (mg/L)	Sample volume ml
5-10	250
10-20	100
20-50	50.0
50-100	25.0

Calculation

$$NH_3 \text{ (mg/L)} = (A-B) \times 14 \times 0.02 \times 1000 / \text{Volume of sample (ml)}$$

where

A = Volume of H₂SO₄, titrated for sample, ml

B = Volume of H₂SO₄, titrated for blank, ml

14 = Molecular weight of nitrogen in ammonia

0.02 = Normality of sulphuric acid

5.11 STANDARD OPERATING PROCEDURE FOR TOTAL KJELDAHL NITROGEN (TKN)

Scope and field of application

- The Kjeldahl method determines nitrogen in the tri-negative state. They fail to account for nitrogen in the form of azide, azine, azo, hydrazine, nitrate, nitrite, nitrile, nitro, nitroso, oximine and semi-carbazone.
- Kjeldahl nitrogen is the sum of organic nitrogen and ammonia nitrogen. Organic nitrogen includes proteins, peptides, nucleic acids and urea.
- The macro-Kjeldahl method is applicable to samples containing high concentrations of organic nitrogen but requires a relatively large sample volume for low concentrations.
- In the semi-micro-Kjeldahl, which is applicable to samples containing high concentrations of organic nitrogen, the sample volume should be chosen to contain organic plus ammonia nitrogen in the range of 0.2–2 mg.

Summary

- In the presence of sulphuric acid (H_2SO_4), potassium sulphate (K_2SO_4), and copper sulphate (CuSO_4) catalyst, amino nitrogen of many organic materials is converted to ammonium.
- Free ammonia also is converted to ammonium.
- After addition of base, the ammonia is distilled from an alkaline medium and absorbed in boric or sulphuric acid.
- The ammonia may be determined colorimetrically, by ammonia-selective electrode, or by titration with a standard mineral acid.
- The titrimetric and selective electrode methods of measuring ammonia in the distillate are suitable for determining a wide range of organic nitrogen concentration.

Interferences

- The most reliable results are obtained on fresh samples.
- If an immediate analysis is not possible, preserve samples for Kjeldahl digestion by acidifying to pH 1.5 to 2.0 with concentrated sulphuric acid and storing at 4°C.
- Do not use HgCl_2 because it will interfere with ammonia removal. HgCl_2 reacts with ammonia to form volatile and potentially harmful compounds, such as mercury ammoniate
- Nitrate: During Kjeldahl digestion, nitrate in excess of 10 mg/L can oxidize a portion of the ammonia released from the digested organic nitrogen, producing N_2O , resulting in negative interference.

- Inorganic salts and solids: The acid and salt content of the Kjeldahl digestion reagent is intended to produce a digestion temperature of about 380°C. If the sample contains a very large quantity of salts or inorganic solids the temperature may rise to 400°C during digestion at which point pyrolytic loss of nitrogen occurs. To prevent this increase in temperature, add more sulphuric acid to maintain an acid-salt balance.

Safety precautions

- Handle concentrated sulphuric acid with care.
- Always use safety goggles, gloves and laboratory coat while working in the laboratory.
- After the analysis clean bottles and beakers with clear water and keep for drying.
- Dispose of used gloves after completing analysis.
- Clean hands using antiseptic soap.
- Disinfect hands after washing with soap.
- Avoid spillage and contact with skin. In the latter case, use copious quantities of cold water to wash skin and call for medical attention.

Apparatus

- Digestion apparatus: Digest over a heating device adjusted so that 250 ml water at an initial temperature of 25°C can be heated to a rolling boil in about 5 minutes. The temperature range should be 375–385°C for effective digestion.
- Distillation apparatus
- 250 ml TKN digestion tubes
- Titration apparatus
- Glass bead
- Erlenmeyer flask
- Cylinder
- Volumetric flask

Reagents

Prepare all reagents and dilution in ammonia-free water. All of the reagents listed for the determination of nitrogen (ammonia), are required, plus the following:

- ***Digestion reagent***

Mix 134 g K_2SO_4 and 7.3 g $CuSO_4$ thoroughly using a mortar and pestle and store in an airtight container until use

- ***Sulphuric acid: 0.1 N***

Dissolve 2.8 ml of concentrated sulphuric acid into 1 L of distilled water

- ***Sodium hydroxide: 35 per cent***

Dissolve 350g of NaOH into 1 L of distilled water

- ***Sodium hydroxide-sodium thiosulphate reagent***

Dissolve 500 g NaOH and 25 g Na₂S₂O₃ 5H₂O in water and dilute to 1 L.

- ***Sodium hydroxide: NaOH, 6N***

- ***Mixed indicator***

Dissolve 200 mg methyl red indicator in 100 ml 95 per cent ethyl or isopropyl alcohol or ethanol. Dissolve 100 mg methylene blue in 50 ml 95 per cent ethyl or isopropyl alcohol or ethanol. Combine solutions. Prepare monthly.

Procedure

Sample preparation

- Weigh out 30 ml of well-mixed faecal sludge sample.
- Place the weighed-out sample into a blender with 270 ml of distilled water.
- Blend for 30 seconds.
- Transfer the blended mixture into a volumetric flask and top up to 1 L with distilled water.
- Transfer the 1 L solution to a plastic bottle and store in the cold room.

Instrument set-up (VELP Scientifica)

- Add 1000 ml of 32 per cent NaOH into reagent bottle 2, screw in bottle (clockwise) and push the bubbling tube to the bottom.
- Follow programme I (150–380°C) where stepwise increase of temperature is set.
- Place suction cap onto tubes and open tap until a steady flow of water is reached (2L/ min).
- Set pump to Mode A, air flow no: 4 until temperature of heating block reaches 200°C.
- Then set pump to Mode B: air flow no: 4 until end of digestion.
- Reactivate Mode B—100 per cent of the maximum air flow—if SO₃ gas emission is too much.

Digestion

- Place 50 ml of mixed diluted sample into 300ml Kjeldahl flask for raw or primary sewage, wastewater or 140 ml for ponds, rivers or final effluents.
- Add a glass rod to the tube, and 5 boiling stones.
- Slowly add 10 ml of concentrated sulphuric acid, 1 Kjeltab or two spatulas powder of digestion reagent (3–5g) and swirl to dissolve. Wait approximately 15 minutes or overnight if sample has a high organic/fat content and then place onto digestion unit.
- Boil briskly at 420°C until dense fumes of SO₃ are evolved and a pale green colour is obtained.
- The required temperature, i.e. 420°C, is usually reached after an hour.
- Keep the suction pump running for 30 minutes after samples are fully digested and heating block is switched off.
- If the sample is too little before it is fully digested, add 10 ml concentrated sulphuric acid and remember to increase the volume of sodium hydroxide used during the distillation.
- Digestion takes about three hours. The colour changes from blue to dark green to black to colourless/pale green.
- Switch off the heating block, pump and the water supply.
- Replace water in the water bath and replace the NaOH in reagent bottle 2.

Distillation

- Prepare absorption solution by placing 50 ml of 2% boric acid with a few drops of mixed indicator in a 250 ml conical flask and insert under the condenser outlet with the tip below the surface of boric acid. The boric acid solution turns green upon collection of distillate.
- Perform the same process with a blank (distilled water).

Enter distillation programme in the instrument as follows:

Volume of water: 100 ml

Volume of NaOH: 50 ml (if 10 ml sulphuric acid used in digestion); 200 ml (if 30 ml sulphuric acid used in digestion)

- Add 50 ml distilled water to the acid digested sample to increase the sample volume and add 3–5 drops of phenolphthalein indicator. This tube is now ready to be kept in the distillation unit for distillation. Switch on the distillation programme entered.

- Sample in tube turns purple with addition of NaOH: indicates pH above 11 before distillation
- Distillation time: 5 minutes

Titration

- Titrate the distillate against 0.1 N sulphuric acid
- Colour change: from green to purple

$$\text{Nitrogen } \left(\frac{\text{mg}}{\text{L}}\right) = \frac{(\text{Titer value of sample} - \text{titer value of blank}) \times 0.1 \times 14 \times 1000}{\text{Sample Volume (ml)}}$$

where:

0.1: Normality of sulphuric acid used in titration

14: Atomic weight of nitrogen

1000: Conversion of g to mg

5.12 STANDARD OPERATING PROCEDURE FOR NITRATE ESTIMATION BY SPECTROPHOTOMETRIC METHOD

Scope and application

Nitrate is a major source of available nitrogen for plants and microorganisms. However, nitrate becomes toxic and affects public health when it contaminates surface or groundwater used as drinking water. Eutrophication and algal blooms of surface water are the aftereffects of nitrate pollution in waterbodies. The method uses a Merck nitrate spectrophotometric test kit for samples with concentrations of 0.1–25 mg NO₃-N/L, which is based on the manufacturer's protocol for water and wastewater using the standard method 4500-NO₃-E.

Summary

In sulphuric and phosphoric solution nitrate ions react with 2,6-dimethylphenol (DMP) to form 4-nitro-2,6-dimethylphenol that is determined photometrically. This test is not suited for determination in waters with chloride contents exceeding 1000 mg/L and COD values exceeding 500 mg/L.

Interferences

Faecal sludge samples and highly turbid wastewater samples can interfere with colour development. Hence, samples must be homogenized, diluted and filtered to avoid interferences. Nitrate measurements are easily influenced by interfering

components such as dissolved organic matter, surfactants, nitrite and various inorganic compounds in water.

Sample preservation

Most reliable results are obtained on fresh samples. If the samples cannot be analysed immediately, they should be preserved at 4°C. Before analysis, samples should be thawed to room temperature.

Safety precautions

Always use safety goggles, gloves and laboratory coats while working in the laboratory. Use eye and hand protection when handling colour reagents. Avoid any spillage of the reagent on the body. If any spillage occurs, wash the area with plenty of water.

Apparatus

- Empty cells 16 mm with screw caps
- Photometer
- Weighing balance
- Blender
- Volumetric flasks
- Pipette and pipette tips
- Glass beakers
- Filter paper

Reagents

- 1 bottle of reagent NO₃-1
- 1 bottle of reagent NO₃-2
- 1 autoselector

Sample preparation

- For slurry to solid samples, weigh out 1.8–2.0 g of thoroughly mixed faecal sludge sample into a beaker.
- Dilute the sample and transfer to a blender for homogenization.
- Transfer the contents of the blender into a 1 L volumetric flask, rinse the blender twice with 250 mL distilled water and dilute to the mark with distilled water.
- Filter the samples through a 0.45 µm filter paper
- Check the chloride content with the MQuant® Chloride Test. Samples containing more than 1000 mg/l Cl⁻ must be diluted with distilled water.

-
- Check the nitrite content with the MQuant® Nitrite Test. If necessary, eliminate interfering nitrite ions (stated amounts apply for nitrite contents of up to 50 mg/l).
 - To 10 ml of sample add approx. 50 mg of amidosulphuric acid and dissolve. The pH of this solution must be within the range of 1–3.
 - Adjust, if necessary, with sulphuric acid.
 - Check the nitrate content with the MQuant® Nitrate Test. Samples containing more than 25.0 mg/l NO₃-N (110.7 mg/l NO₃-) must be diluted with distilled water.

Procedure

- Pipette 4 ml of reagent NO₃-1 into a dry test tube.
- Add 0.5 ml of sample into the above solution. Do not mix.
- Add 0.5 ml of reagent NO₃-2 and mix. The mixture becomes hot.
- Leave the hot reaction solution to stand for 10 min (reaction time). Do not cool with cold water.
- Analyse the above solution using a photometer (Concentration of NO₃- in mg/L will be directly displayed in the photometer).
- To increase the accuracy, analysis of blank can be carried out in the same manner. (Add the reagents to distilled water instead of sample.)
- Select the respective Programme in the spectroquant photometer.
- Measure the sample in the photometer. The results are displayed as mg/L.

Result Interpretation

The concentration of nitrate in mg/L can be directly read from Spectroquant photometer. If the sample is diluted before or during analysis, then dilution factor has to be multiplied with the photometer reading

5.13 STANDARD OPERATING PROCEDURE FOR NITRITE ESTIMATION BY SPECTROPHOTOMETRIC METHOD

Scope and application

This method describes nitrite measurement by spectrophotometry. Spectrophotometric measurements ranging from 0.01–1.0 mg/L are applicable for this method. However, for lower concentrations < 0.01 mg/L, spectrophotometric measurements can be made by adapting this method using a 5 cm light path and a green colour filter. Merck Spectroquant Nitrite Test spectrophotometric test kit allows the determination of samples with concentrations of 0.002–1.0 mg NO₂-N/L, and it is based on the manufacturer's protocol for water and wastewater.

Summary

The principle is that in acidic solution nitrite ions react with sulphanilic acid to form a diazonium salt. The diazonium salt reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red-violet azo dye at a pH of 2.0-2.5, which is then determined spectrophotometrically.

Interferences

Faecal sludge samples and highly turbid wastewater samples can interfere with the spectrophotometric determination. Hence, samples must be homogenized, diluted and filtered to avoid the interferences. For spectrophotometric measurements, the cuvettes must be clean. Common interferences in faecal sludge include magnesium and nitrates. For specific concentrations refer to the manufacturer's instructions.

Preservation

Most reliable results are obtained on fresh samples. If the samples cannot be analysed immediately, they should be preserved at 4°C. Before analysis, samples should be thawed to room temperature.

Safety Precautions

Always use safety goggles, gloves and laboratory coat while working in the laboratory. Eye and hand protection when handling color reagents. Avoid any spillage of the reagent on the body. If any spillage occurs, wash the area with plenty of water.

Apparatus

- Spectrophotometer equipped with 1 cm or larger cuvettes,
- Weighing balance,
- Blender,
- Volumetric flasks,
- Pipette and pipette tips,
- Glass beakers,
- Filter paper,
- pH test strip,
- Blue micro spoon (provided by the manufacturer)

Reagents

- Reagent NO₂-1 (supplied by the manufacturer),
- Nitrite standard solution CRM
- 0.200 mg/L NO₂-N (supplied by the manufacturer)

Sampling

- Samples should be analysed immediately after sampling to prevent the bacterial conversion of NO_2^- to NO_3^- or NH_3 .

Sample preparation

- Samples containing concentrations of nitrite beyond the range of the test kit must be diluted with distilled water. The nitrate content of samples can be estimated before dilution with the MQuant nitrite test kit. Filter the samples through a 0.45 μm filter paper.
- For slurry to solid samples, weigh out between 1.8 g and 2.0 g of thoroughly mixed faecal sludge sample into a beaker.
- Dilute the sample and transfer to a blender for homogenization.
- Transfer the contents of the blender into a 1 L volumetric flask, rinse the blender twice with 250 mL distilled water and dilute to the mark with distilled water.
- Filter the samples through a 0.45 μm filter paper and measure the nitrate concentration according to the procedure.

Calibration

Follow the spectrophotometer manufacturer's instructions for calibration, since calibration procedure differs between instruments. It may be necessary to calibrate the instrument before every reading, or it may only be necessary to perform periodic calibration checks to determine when calibration is necessary.

Analysis procedure

- Pipette 5ml of the sample into a test tube.
- Add 1 level blue micro-spoon of reagent NO_2^-1 to the test tube.
- Shake vigorously until the reagent is completely dissolved.
- The pH of the solution must be between 2-2.5; check this with a pH test strip.
- Leave the solution to stand for 10 min, then fill the 10 mm cuvette with the sample.
- Select the respective programme in the spectroquant photometer.
- Wipe the cuvette with a soft tissue to remove water spots and fingerprints and then measure in a spectrophotometer (Spectroquant NOVA60).
- The results are displayed as mg/L.

Result interpretation

The concentration of nitrite in mg/L can be directly read from Spectroquant photometer. If the sample is diluted before or during analysis, then dilution factor has to be multiplied with the photometer reading

5.14 STANDARD OPERATING PROCEDURE FOR ORTHO-PHOSPHATE AND TOTAL PHOSPHATE

Scope and application

The measurement of total phosphorus and phosphate is essential for performance studies of wastewater, faecal sludge and sewage treatment plants.

Principle

Colorimetric method: In sulphuric solution, orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically.

Interferences

- Sample for phosphate analysis must be pretreated by filtration (0.45µm) to remove most of the turbidity (interferes with photometric measurement).

Sampling

Collect samples preferably in glass bottles.

Safety precautions

- Handle concentrated acids with care.
- Always use safety goggles, gloves and laboratory coat while working in laboratory.
- After the analysis, clean bottles and beakers with clear water and keep them for drying.
- Dispose of the used gloves after completing analysis.
- Clean hands using antiseptic soap.
- Disinfect hands after washing with soap.
- Avoid spillage and contact with skin. In the latter case, use copious washings with cold water and call for medical attention.

Apparatus

- Heating block for total P measurement.
- Spectrophotometer.
- Glassware: Use acid-washed glassware for determining low concentrations of orthophosphates.

Note: Phosphate contamination is common because of its adsorption on glass surfaces. Avoid using commercial detergents containing phosphate. Clean all glassware with hot dilute HCL and rinse well with distilled water. Preferably

reserve the glassware only for phosphate determination and after use, wash and keep filled with water until needed. If this is done, acid treatment is required only occasionally.

Reagents

For digestion/pretreatment of the samples (Spectroquant crack set 10)

- Reagent R1
- Reagent R2
- Reagent R3

Phosphate test

- Reagent PO₄ - 1
- Reagent PO₄ - 2

Calibration

To check the photometric measurement system (test reagents, measurement device and handling) and the mode of working, Spectroquant® Combi Check 10 can be used. Besides a standard solution with 0.80 mg/L PO₄-P, Combi Check 10 also contains an additional solution for determining sample-dependent interferences (matrix effects).

Sample preparation

- Filter paper dimensions: diameter = 47 mm, pore size = 0.45 microns
- Filter the diluted solution using a Buchner funnel.
- Collect the filtrate for analysis.

Procedure

Note: Procedures according to Merck operational Manual for test kits (phosphate 1.14848.0001 and total P 1.14543.0001)

Ortho-phosphate measurement

- Pipette 5.0 ml pretreated (diluted and filtered) sample into a test tube.
- Add 5 drops of reagent PO₄-1 and mix.
- Add 1 level blue micro spoon of reagent PO₄ - 2 and shake vigorously until the reagents are completely dissolved.
- Leave to stand for five minutes (reaction time), then transfer the sample into the cell or cuvette for spectrophotometric measurement directly read as concentration the sample.

Total phosphorous measurement

- Digestion for the determination of total phosphorus (wear eye protection!).
- Pipette 10.0 ml pretreated sample into a reaction cell.
- Add 1 drop Reagent R1 and 1 dose R2, close the cell tightly, and mix.
- Heat the cell at 120°C in the preheated thermo reactor for one hour.
- Allow the closed cell to cool to room temperature in a testtube rack. Do not cool with cold water!
- Shake the tightly closed cell vigorously after cooling.
- Add 3 drops of reagent R3, close the cell tightly, and shake vigorously until the reagent is completely dissolved.
- Pipette 5 ml of digested sample into fresh cell, then add 5 drops of reagent PO₄-1 and mix.
- Add 1 level blue micro spoon of reagent PO₄ - 2 and shake vigorously until the reagents completely dissolved.
- Leave to stand for five minutes (reaction time).
- Select the respective programme in the spectroquant photometer
- Measure the concentration using the photometer.

Result interpretation

The concentration of phosphate in mg/L can be directly read from Spectroquant photometer. If the sample is diluted before or during analysis, then dilution factor has to be multiplied with the photometer reading.

5.15 STANDARD OPERATING PROCEDURE FOR BIOLOGICAL OXYGEN DEMAND (BOD)

Scope and application

Biochemical oxygen demand (BOD) is an empirical standardized laboratory test that measures oxygen requirement for aerobic oxidation of decomposable organic matter and certain inorganic materials in water, polluted waters and wastewater under controlled conditions of temperature and incubation period. The quantity of oxygen required for above oxidation processes is a measure of the test. The test is applied for freshwater sources (rivers, lakes), wastewater (domestic, industrial), polluted receiving waterbodies, marine water (estuaries, coastal water) for finding out the level of pollution, assimilative capacity of waterbody and performance of waste treatment plants.

Principle

Titrimetric method

This test measures the oxygen utilized for the biochemical degradation of organic material (carbonaceous demand) and oxidation of inorganic material such as sulphides and ferrous ions during a specified incubation period. It also measures the oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. Temperature effects are held constant by performing a test at fixed temperature. The methodology of BOD test is to compute a difference between initial and final DO of the sample's incubation. A minimum of 600 ml of sample is required for the test. DO is estimated by iodometric titration.

Since the test is mainly a bio-assay procedure, it is necessary to provide standard conditions of temperature, nutrient supply, pH (6.5–7.5), adequate population of microorganisms and absence of microbial-growth-inhibiting substances. The low solubility of oxygen in water necessitates strong wastes to be diluted to ensure that the demand does not exceed the available oxygen. A mixed group of microorganisms should be present in the sample; otherwise, the sample has to be seeded. Generally, temperature is controlled at 20°C and the test is conducted for five days, as 70–80 per cent of the carbonaceous wastes are oxidized during this period. The test can be performed at 20°C for five days (BOD₅) or at 27°C for three days (BOD₃) in Indian conditions.

Interference

Since DO estimation is the basis of BOD test, sources of interference in BOD test are the same as in the DO test. In addition, lack of nutrients in dilution water, lack of an acclimated seed organisms and presence of heavy metals or other toxic materials such as residual chlorine are other sources of interferences in this test. Adjust the sample pH to 7.5. Remove high residual chlorine by adding predetermined sodium sulphate solution.

Equipment and apparatus

- BOD bottles 300 ml capacity (clean with a detergent, rinse thoroughly and drain before use) with a water seal.
- Incubator or water-bath to be controlled at 20°C or at any desired temperature. Exclude all light to prevent photosynthetic production of DO.

Reagents and standards

- Manganese sulphate: Dissolve 480 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ or 400g $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ in distilled to 1000 ml. Filter if necessary. This solution should not give colour with starch when added to an acidified solution of KI.
- Alkali iodide-azide reagent: For saturated or less than saturated samples: Dissolve 500g NaOH (or 700g KOH) and 150g KI (or 135g NaI) in distilled water and dilute to 1000ml. Add 10g sodium azide, NaN_3 dissolved in 40 ml distilled water. This solution should not give colour with starch solution when diluted and acidified.
- For supersaturated samples: Dissolve 10 g NaN_3 in 500 ml distilled water. Add 480 g NaOH and 750g NaI and stir to dissolve the contents.

Cautions: Do not acidify this solution because toxic hydrozoic acid fumes may be produced.

- Sulphuric acid: H_2SO_4 , conc., 1 ml is equivalent to about 3ml alkali-iodide-azide reagent.
- Starch indicator: Prepare a paste or solution of 2.0 g of soluble starch powder as well as 0.2 g salicylic acid as preservative in distilled water. Pour this solution in 100 ml boiling distilled water. Continue boiling for a few minutes, cool and then use.
- Stock sodium thiosulphate, 0.1 N: Dissolve 24.82 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in distilled water. Preserve by adding 0.4 g solid NaOH or 1.5 ml of 6N NaOH and dilute to 1000 ml.
- Standard sodium thiosulphate, 0.025N: Dilute 250ml stock $\text{Na}_2\text{S}_2\text{O}_3$ solution to 1000 ml with freshly boiled and cooled distilled water. Add preservative before making up the volume.

Note: This should be standardized with standard dichromate solution for each set of titrations.

- Phosphate buffer: Dissolve 8.5 g KH_2PO_4 , 21.75g K_2HPO_4 , 33.5g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 1.7g NH_4C ; in distilled water and dilute to 1000 ml. The pH should be 7.2 without further adjustment. Discard reagent if there is any sign of biological growth.
- Magnesium sulphate: Dissolve 22.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in about 700 ml of distilled water and dilute to 1 L.
- Calcium chloride: Dissolve 27.5 g anhydrous CaCl_2 in about 7000 ml of distilled water and dilute to 1 L.
- Ferric chloride: Dissolve 0.25g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in about 700 ml of distilled water and dilute to 1 L.

- Sodium sulphate solution 0.025N: Dissolve 1.575g Na_2SO_3 in distilled water and dilute to 1000 ml. Solution should be prepared daily.
- Acid and alkali solutions 1N: Prepare 1N H_2SO_4 and 1N NaOH or neutralization of caustic or acidic samples.
- Nitrification inhibitor: 2-chloro-6-(trichloromethyl) pyridine (nitrification inhibitor 2570-24 [2.2% TCMP])

Procedure

Preparation of dilution water

- The source of dilution water may be distilled water, tap or receiving-stream water free of biodegradable organics and bio-inhibitory substances such as chlorine or heavy metals.
- Aerate the required volume of dilution water in a suitable bottle by bubbling clean- filtered compressed air for sufficient time (one to two hours) to attain DO saturation at room temperature or at 20°C/27°C.
- Before use stabilize the water at 20°C/27°C.
- Add 1 ml each of phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride solutions, in this order, for each litre of dilution water. Mix well. The quality of dilution water may be checked by incubating a BOD bottle full of dilution water for five days at 20°C for three days at 27°C. DO uptake of dilution water should not be more than 0.2 mg/L and preferable not more than 0.1 mg/L.
- For wastes that are not expected to have sufficient microbial population, seed is essential. Preferred seed is effluent from a biological treatment system. Where this is not available, supernatant from domestic wastewater (domestic sewage) settled at room temperature for at least one hour but not longer than 36 hours is considered sufficient in the proportion 1–2 ml/L of dilution water. Adopted microbial population can be obtained from the receiving water body preferably 3–8 km below the point of discharge. In the absence of such situation, develop an adapted seed in the laboratory.
- Determine BOD of the seeding material. This is seed control. From the value of seed control determine seed DO uptake. The DO uptake of seeded dilution water should be between 0.6 mg/L and 1 mg/L.

Sample preparation

- Neutralize the sample to pH 7, if it is highly acidic or alkaline.
- The sample should be free from residual chlorine. If it contains residual chlorine remove it by using $\text{Na}_2\text{S}_2\text{O}_3$ solution as described below.
- Take 50 ml of the sample and acidify with addition of acetic acid. Add about 1g

KI. Titrate with 0.025N $\text{Na}_2\text{S}_2\text{O}_3$, using starch indicator. Calculate the volume of $\text{Na}_2\text{S}_2\text{O}_3$ required per litre of the sample and accordingly add to the sample to be tested for BOD.

- Certain industrial wastes contain toxic metals, e.g. planting wastes. Such samples often require special study and treatment.
- Bring samples to $20 \pm 1^\circ\text{C}$ before making dilutions.
- If nitrification inhibition is desired, add 3 mg 2-chloro-6-(trichloromethyl) pyridine (TCMP) to each 300 ml bottle before capping or add sufficient amount to the dilution water to make a final concentration of 30 mg/L. Note the use of nitrogen inhibition in reporting results.
- In samples having high DO contents, DO >9 mg/L should be treated to reduce the DO content to saturation at 20°C . Agitate or aerate with clean, filtered compressed air.

Dilution of sample

Dilutions that result in a residual DO of at least 1mg/L and DO uptake of at least 2 mg/L produce reliable results. Make several dilutions of the pre-treated sample so as to obtain about 50 per cent depletion of DO or DO uptake of 2 mg/L. Prepare dilutions as follows:

- Siphon out half the required volume of seeded dilution water in a graduated cylinder or volumetric flask without entraining air. Add the desired quantity of mixed sample and dilute to the appropriate volume by siphoning dilution water. Mix well with plunger type mixing rod to avoid entraining air.
- Sample processing.
- Siphon the diluted or undiluted sample in three labelled bottles and stopper immediately.
- Keep one bottle for determination of the initial DO and incubate two bottles at 27°C for three days. See that the bottles have a water seal.
- Prepare a blank in triplicate by siphoning plain dilution water (without seed) to measure the O_2 consumption in dilution water.
- Also prepare a seed blank in triplicate to measure BOD of seed for correction of actual BOD.
- Determine DO in a BOD test of the blank on initial day and end of incubation period by Winkler method as described for DO measurement.
- DO estimation in a BOD test can also be done by membrane electrodes. A DO probe with a stirrer is used to determine initial and final DO after incubation in BOD samples. The semi-permeable membrane provided in the DO probe acts as a diffusion barrier against impurities between sensing element and sample.

DO measurement

- Collect sample in a BOD bottle.
- Add 1 ml MnSO_4 followed by 1ml of alkali-iodide-azide reagent to the sample collected in 250 to 300 ml bottle up to the brim. The tip of the pipette should be below the liquid level while adding these reagents. Stopper immediately. Rinse the pipettes before putting them to reagent bottles.
- Mix well by inverting the bottle two to three times and allow the precipitate to settle leaving 150 ml clear supernatant. The precipitate is white if the sample is devoid of oxygen, and becomes increasingly brown with rising oxygen content.
- At this stage, add 1 ml conc. H_2SO_4 . Replace the stopper and mix well till precipitate goes into solution.
- Take 201 ml of this solution in a conical flask and titrate against standard $\text{Na}_2\text{S}_2\text{O}_3$ solution using starch (2 ml) as an indicator. When 1 ml MnSO_4 followed by 1 ml alkali- iodide-azide reagent is added to the samples as in above, 2 ml of original sample is lost. Therefore 201 ml is taken for titration which will correspond to 200mL of original sample.

Note: $200 \times 300 / (300 - 2) = 201 \text{ ml}$

Calculation

1ml of 0.025N $\text{Na}_2\text{S}_2\text{O}_3 = 0.2 \text{ mg of O}_2$

DO in mg/L = $(0.2 \times 1000) \times (0.025\text{N}) \text{ ml of thiosulphate} / 200$, when dilution water is not added

BOD as O_2 mg/L = $(D1 - D2) \times 1000 / \% \text{ dilution}$

O_2 mg/l = $(D1 - D2) - (B1 - B2) \times 1000 / \% \text{ dilution}$

Where,

D1 = DO of sample immediately after preparation, mg/L
D2 = DO of sample after incubation period, mg/L

B1 = DO of blank (seeded dilution water) before incubation, mg/L
B2 = DO of blank (seeded dilution water) after incubation, mg/L

5.16 STANDARD OPERATING PROCEDURE FOR BIOLOGICAL OXYGEN DEMAND (BOD) USING RESPIROMETRIC METHOD

Principle

The BOD measuring unit, comprising a test bottle and BOD sensor, is a closed system. With the filled sample quantity, there is a gas compartment with a defined quantity of air in the test bottle. The bacteria in the wastewater filled in the bottle (the sample can be used diluted or undiluted) consume the oxygen dissolved in the sample over the course of the BOD measurement. It is replaced by air oxygen from the gas compartment of the test bottle. The simultaneously developing carbon dioxide is chemically bound by the potassium hydroxide in the seal cup of the test bottle. As a result, a pressure drop occurs in the system, which is measured by the BOD sensor and shown directly in the display as a BOD value in mg/L O₂.

Equipment

LOVIBOND BD 600

Chemicals and consumables

Potassium hydroxide solution, nitrification inhibitor

Materials required

BOD base unit with integrated bottle rack, BOD bottles, BOD sensor, seal cup, magnetic stir bar, volumetric flasks

Procedure

Selection of sample volume

The volume of sample required for analysis depends upon the expected BOD range. The table 4 provided below displays the expected BOD range and the volume of sample that must be taken in the bottle.

Table 4: Selection of sample volume and nitrification inhibitor

BOD range in mg/L	Sample volume in ml	Nitrification inhibitor ATH dosage
0-40	428	10 drops
0-80	360	10 drops
0-200	244	5 drops
0-400	157	5 drops
0-800	94	3 drops
0-2000	56	3 drops
0-4000	21,7	1 drop

Preparation of the water sample

- Depending on the specifications, the water sample must be mixed well, briefly allowed to settle, and filtered or homogenized.
- Determine the pH of the water sample and check if it lies in the range 6.5–7.5. If the pH is not within this range, then add acid or base to adjust the pH.
- Measure the exact necessary sample quantity as mentioned in the table with the appropriate overflow volumetric flask and add it to the test bottle.
- Add the nitrification inhibitor to the sample.
- Add a clean magnetic stir bar to each test bottle and fill the dry seal cup with three or four drops of 45 per cent potassium hydroxide solution (for binding of the carbon dioxide). Then place the sealed cup in the test bottle.
- The prepared sample must be brought to ± 1 °C of the desired temperature before the start of the measurement (e.g. 20 °C \pm 1 °C). Switch 'On' thermostat cabinet to set the desired temperature.
- Place the BOD sensors on the test bottles and carefully screw them in place. This is especially important because the system must be tight. Then place the BOD bottle with the sensor screwed on in the bottle rack. This can be done directly in the thermostat cabinet.
- Start the test and incubate the sample according to specifications.
- To start the test, switch 'On' instrument display.
- In the display, 'Main menu' is divided into five submenus namely 'Start measurement series', 'display current values', 'display measurement series', 'export measurement series' and 'options'.
- Select the first option 'Start measurement series'. This menu is further divided into three: 'Measurement position' (bottle position) in the cabinet, 'Measurement range' (expected BOD range), 'Measurement duration'. Fill in the details in all three for all the samples placed in the cabinet.
- Then start a series of measurement.

Result interpretation

Results will be displayed in the 'display current values' section. BOD value is obtained as mg/L O₂. Before noting the values, press 'update' so that the updated measured values will be displayed.

5.17 STANDARD OPERATING PROCEDURE FOR TESTING OF FAECAL COLIFORM IN FAECAL SLUDGE AND WASTEWATER BY MOST PROBABLE NUMBER (MPN) METHOD

1. Scope and application

Coliforms are a group of bacteria that are predominantly found in the gastrointestinal tract of warm-blooded animals including humans. They are also present in soils, surface waters and on plant surfaces. Specific genera included in this group are *Escherichia*, *Klebsiella* and *Enterobacter*. These bacteria are facultatively anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that are able to ferment lactose and produce acid and gas within 24 hours at 35°C. Faecal coliforms (FC) (thermotolerant coliforms) are specific group of coliform bacteria that have the ability to grow, ferment lactose and produce acid and gas at elevated temperatures. These FC bacteria are exclusively found in the intestines and faeces of humans and other warm-blooded mammals and hence their presence indicates faecal contamination. The predominant FC is *Escherichia coli*. *E. coli* is a typical FC which from ancient times is used as an indicator organism to specifically indicate faecal contamination of various sources including drinking water, surface water, groundwater and wastewater. Generally, it is expected that the density of these bacteria directly correlates with the probability of pathogen presence. The ease of cultivation and detection made these bacteria the standard indicator organisms in sanitation. The use of these bacteria as fecal indicators can be extended to faecal sources like FS, FS biosolids, FS liquid effluent etc. to estimate the densities of FC/*E. coli* present in these samples.

2. Principle

The multiple tube fermentation (MTF) method using A-1 medium is a direct single step test used for the detection and enumeration of faecal coliform bacteria from samples like source water, treated wastewater (APHA, 2023), biosolids (US EPA, 2006) or faecal sludge where the possibility of the presence of faecal coliforms is moderately high. Hence, prior enrichment in presumptive medium is not required. The culture-specific growth medium used in this method has lactose as a fermentable carbohydrate which is utilized by faecal coliform bacteria for growth to produce acid and gas within 24 h of incubation at elevated temperatures ($44.5 \pm 2^\circ\text{C}$). The gas production is considered as a positive test which is detected in the inverted Durham tubes present in the medium.

This method is a quantitative method which uses standard serial dilution technique to estimate the densities of organisms present in the sample. In serial dilution procedure, the sample is diluted serially depending on the bacterial load present in the sample. Dilutions of the sample are then selected (minimum 3 dilutions) and each dilution is inoculated into multiple (replicate) tubes with desired medium (A-1) and incubated at optimum temperature ($44.5 \pm 2^\circ\text{C}$) and observed for positive result (growth and gas production) within the specified time period (24 ± 2 h). The total number of positive tubes in each dilution are recorded. From the positive results in different dilutions, three significant dilutions are selected based on the guidelines given in the MPN procedure. The combinations of positive results at selected significant dilutions are matched with the same series present in the statistical reference MPN chart to obtain the most probable number (MPN) of the organisms present in the sample. All the dilutions made to the sample are taken into account for calculating the density of organisms (FC) present in the sample.

3. Apparatus

- Latex gloves;
- Conical flask, 2 L;
- Measuring cylinders (1 L, 50 ml);
- Weighing balance;
- Butter paper;
- Microwave oven/hot plate;
- Screw cap bottle, borosilicate glass, 500 ml;
- Culture media dispensing pump;
- Test tubes, rimless, borosilicate glass (18×150 mm, 15×150 mm);
- Loose-fitting autoclavable plastic caps (18 mm, 15 mm diameter);
- Durham tubes, borosilicate glass, 10×60 mm;
- Polygrid test tube stand, autoclavable (16 mm, 20 mm diameter);
- Test tube basket, autoclavable, 180 x 170 x 160 mm;
- Autoclave;
- Laboratory blender;
- Wide mouth bottle, polypropylene, autoclavable, 500 ml;
- Biosafety cabinet/laminar airflow;
- Bunsen burner or alcohol burner;
- Vortex mixer; micropipettes and tips;
- Incubator/water bath.

4. Reagents

Distilled water; A-1 broth (HiMedia)

5. Reagent preparation

5.1 Culture media

- To prepare 1 L of A-1 media, take 1 L of distilled water using a 1 L measuring cylinder, and add 500 mL into a 2 L conical flask. Weigh 31.5 g of dehydrated A-1 media powder on a clean butter paper and add it into the 2 L conical flask containing 500 mL of distilled water and dissolve the media completely. Heat if necessary to dissolve. Add the remaining 500 mL of distilled water into the conical flask and mix to form a uniform solution.
- Pour the A-1 media broth into the 500 mL screw cap bottle fitted with a culture media dispensing pump and dispense 10 ml portions of medium into 15 × 150 mm test tubes using the culture media dispensing pump.
- Insert a Durham tube in inverted position into each test tube containing A-1 media and make sure that the inner Durham tube is completely filled with media leaving no air spaces.
- Close the tubes with 15 mm autoclavable loose fitting plastic caps, place them in a test-tube basket or test-tube stand and sterilize by autoclaving at 121°C (15 PSI pressure) for 15 minutes.

5.2 Water for serial dilution

- Pour distilled water into the 500 mL screwcap bottle fitted with a culture media dispensing pump.
- Dispense 9 ml of distilled water into 18 mm x 150 mm tubes using the culture media dispensing pump.
- Close the tubes with 18 mm autoclavable loose fitting plastic caps, place them in a test tube basket or test tube stand and sterilize in an autoclave at 121°C temperature (15 PSI pressure) for 15 minutes.

6. Procedure

6.1 Sample preparation/ sample homogenization

- As the FS sample is largely heterogeneous, it has to be homogenized before taking it for analysis. Take the sample bottle containing FS and mix it thoroughly for several (25–30) times.
- After mixing, take 50 mL of the FS sample in a 50 mL measuring cylinder and add it into the sterile laboratory blender jar.
- Take 450 mL of sterile distilled water in a sterile 1 L measuring cylinder.
- Using the sterile distilled water from the 450 mL taken in the 1 L measuring cylinder in the previous step, rinse and transfer entire contents of the sample from the 50 mL measuring cylinder into the blender jar.

- Add the remaining sterile distilled water from the 1 L measuring cylinder into the blender jar, close the jar with lid and blend at high speed for 1–2 minutes. This is the ‘homogenized’ sample. Each mL of the ‘homogenized’ sample contains 10^{-1} (0.1) mL of the original sample.
- Note: For other samples like FS liquid effluent, wastewater, etc. which are mostly homogenous, the homogenization step is not required.

6.2 Sample dilution

- The determination of FC by MPN method requires prior dilution of the samples. The extent of dilution depends on the type of sample being analysed. In the FS sample, the concentration of FC depends on several factors (age of sludge etc. among others). Whereas in the treated FS liquid effluent and treated wastewater, the FC count will vary depending on the extent of treatment. Sometimes, it may be required to dilute the sample several fold (up to 10^{-6} or more in FS sample or any inlet sample of wastewater treatment) to accurately estimate the density of FC.
- For FS sample, take the ‘homogenized sample’ bottle and shake it vigorously several (25–30) times. Take 1 mL aliquot from this bottle and add to tube containing 9 ± 0.2 mL sterile dilution water and mix by vortexing. This tube contains ‘ 10^{-2} mL’ of original sample. Label this dilution as ‘ 10^{-2} ’.
- Take 1 ml aliquot from the tube with ‘ 10^{-2} mL’ of original sample and add to sterile tube containing 9 ± 0.2 mL sterile dilution water and mix by vortexing. This tube contains ‘ 10^{-3} mL’ of original sample. Label this dilution as ‘ 10^{-3} ’. Depending on requirement, serially dilute the sample as done in this step and label the dilutions as ‘ 10^{-4} ’, ‘ 10^{-5} ’, ‘ 10^{-6} ’, ‘ 10^{-7} ’ and so on.
- Do not suspend sample in dilution water for more than 30 min at room temperature to avoid death or multiplication of microorganisms.
- Use sterile pipette and tips at each step of dilution and inoculation to avoid cross contamination.
- Note: For samples other than FS like the FS liquid effluent or wastewater, as the homogenization step is absent, the original sample is directly taken for serial dilution instead of homogenized sample.

6.3 A-1 media test

- If media tubes are kept in a refrigerator after autoclaving, bring all tubes to room temperature (RT) before inoculation by keeping them outside at RT for 1–1.5 h.
- In the present procedure, three replicate tubes per dilution (sample volume/sample quantity) are used. Hence, this method is also referred as ‘3-replicate tube method’.

- Arrange tubes with A-1 medium in rows of three tubes each in a test tube rack.
- Label media name, sample ID, sample dilution (10^{-1} , 10^{-2} , 10^{-3} and so on) and replicate number (10^{-1} .I, 10^{-1} .II, 10^{-1} .III, and 10^{-2} .I, 10^{-2} .II, 10^{-2} .III and so on) on each row of tubes before inoculation.
- Add 1 ml of sample from 10^{-1} dilution tube ('homogenized sample bottle' in case of FS sample; ' 10^{-1} dilution tube' in case of other samples like FS treated effluent, wastewater etc.) to each of three tubes in a row each containing 10 ml of A-1 medium and shake gently so that no air enters inside the Durham tube. Similarly, repeat inoculations for all the dilutions.
- Incubate the inoculated tubes with A-1 medium in an incubator at 35 ± 0.5 °C for $3 \text{ h} \pm 15 \text{ min}$.
- After 3 h of incubation at 35 °C, incubate the tubes in an incubator/ water bath at 44.5 ± 0.2 °C for an additional 21 ± 2 h. If water bath is used, the water level should be above the media in immersed tubes. Total incubation time should be 24 ± 2 h.
- After incubation, remove the tubes from incubator/ water bath and check each tube for the presence of growth (turbidity) and gas production inside the Durham tube. If gas production is not readily apparent inside the Durham tube, shake the tubes gently and check for rising gas bubbles. Growth with gas production is considered as positive result for the presence of FC.
- Note down the positive (indicated by +)/negative (indicated by -) result in each replicate tube of all dilutions of the sample as shown in Table 5 below.
- Count and note down the total number of positive tubes in each dilution for FC (gas production) as shown in the Table 5.
- The positive results of A-1 media (Table 5) are taken for estimation/calculation of FC density according to the procedure shown below in Section 7.
- Note: In case of treated FS liquid effluent/ treated wastewater, direct sample (labelled as ' 10^0 ' which contains '1 mL' of original sample) can also be inoculated in 3 replicate tubes along with dilutions.

Table 5: Recording of results in A-1 media (example)

Sample dilution	10-1		10-2		10-3		10-4		10-5	
	T	GP	T	GP	T	GP	T	GP	T	GP
EML2023xxxx	T	GP	T	GP	T	GP	T	GP	T	GP
Replicate 1	+	+	+	+	+	+	+	+	-	-
Replicate 2	+	+	+	+	+	+	+	-	-	-
Replicate 3	+	+	+	+	+	+	+	+	+	+
Total number of positive tubes in each dilution		3		3		3		2		1

T: turbidity; GP: gas production

7. Data analysis and calculations

- The estimated density of faecal coliform/*E. coli* bacteria, based on the A-1 media test results, is calculated in terms of most probable number (MPN).

7.1 Selection of significant dilutions

- When more than three dilutions of a sample are used in a decimal series of dilutions of a 3-replicate tube MPN determination (as shown in Tables 5 and 6); the following guidelines should be followed.
- Results from only three consecutive dilutions are used to determine the MPN.
- If one or more dilutions have all tubes positive, select the highest dilution (smallest sample quantity) with positive results in all tubes and the next two higher dilutions (see examples 'a' and 'b' in Table 6: *Example calculations including the selection of significant dilutions*).
- When none of the dilutions yield all tubes positive, select the three lowest dilutions for which the middle dilution contains the positive result (see example 'c' in Table 6: *Example calculations including the selection of significant dilutions*).
- If a positive result occurs in a higher unselected dilution, add the number of positive tubes in this dilution to the results of the highest dilution of the three selected (see example 'd' in Table 6: *Example calculations including the selection of significant dilutions*).
- When all dilutions tested yield all tubes positive, select the three highest dilutions (see example 'e' in Table 6: *Example calculations including the selection of significant dilutions*).

Table 6: Example calculations including the selection of significant dilutions

Example	Sample quantities (g or ml) ¹					Reported positive values	MPN estimate/g or ml
	10	1	0.1	0.01	0.001		
a	3/3 ²	<u>3/3</u>	<u>2/3</u>	<u>0/3</u>	0/3	3-2-0	9.3
b	3/3	3/3	<u>3/3</u>	<u>2/3</u>	<u>0/3</u>	3-2-0	93.
c	0/3	<u>0/3</u>	<u>1/3</u>	<u>0/3</u>	0/3	0-1-0	0.31
d	3/3	<u>3/3</u>	<u>2/3</u>	<u>1/3</u>	1/3	3-2-2	21.
e	3/3	3/3	<u>3/3</u>	<u>3/3</u>	<u>3/3</u>	3-3-3	>1100

¹The analyst should make sure that all sample dilution factors (including the preparation of any sample homogenate) are correctly applied in calculating the actual sample quantities subjected to MPN analysis.

$$\begin{array}{l}
 \text{b}^2 \text{ Numerator} \\
 \hline
 \text{Denominator}
 \end{array}
 =
 \begin{array}{l}
 \text{No. positive tubes} \\
 \hline
 \text{No. tubes inoculated}
 \end{array}$$

7.2 Calculation of MPN / mL and MPN / 100 mL

- After selecting the significant dilutions/ valid series, MPN/mL is determined by matching the selected series (also called combination of positives) with the same series (combination of positives) on the MPN reference chart given in Table 7 below.
- If the selected series (combination of positives) does not match the sample dilution series given in the title of the MPN reference chart given in Table 7 below, the results must be calculated using the following formula:
- $MPN / mL = MPN \text{ from chart in Table 7} \times (\text{ml sample for first column of chart} / \text{ml sample in first dilution of the selected series})$
- $MPN / 100 \text{ mL} = MPN \text{ from chart in Table 7} \times (\text{ml sample for first column of chart} / \text{ml sample in first dilution of the selected series}) \times 100$ (As the unit of MoEF&CC standards of India for the discharge of treated water from STPs is given in MPN / 100 mL, the result is calculated and expressed in MPN / 100 mL).

Table 7: MPN Index and 95 per cent confidence limits for various combinations of positive tubes in a 3-tube dilution series using inoculum quantities of 1, 0.1 and 0.01g (ml) of original sample (3-replicate tube method)

Combination of positives	MPN index per g (ml)	95% Confidence limits	
		Lower	Upper
0-0-0	<0.3	---	0.95
0-0-1	0.30	0.015	0.96
0-1-0	0.30	0.015	1.1
0-1-1	0.61	0.12	1.8
0-2-0	0.62	0.12	1.8
0-3-0	0.94	0.36	3.8
1-0-0	0.36	0.017	1.8
1-0-1	0.72	0.13	1.8
1-0-2	1.1	0.36	3.8
1-1-0	0.74	0.13	2.0
1-1-1	1.1	0.36	3.8
1-2-0	1.1	0.36	4.2
1-2-1	1.5	0.45	4.2
1-3-0	1.6	0.45	4.2
2-0-0	0.92	0.14	3.8
2-0-1	1.4	0.36	4.2
2-0-2	2.0	0.45	4.2
2-1-0	1.5	0.37	4.2
2-1-1	2.0	0.45	4.2
2-1-2	2.7	0.87	9.4

Combination of positives	MPN index per g (ml)	95% Confidence limits	
		Lower	Upper
2-2-0	2.1	0.45	4.2
2-2-1	2.8	0.87	9.4
2-2-2	3.5	0.87	9.4
2-3-0	2.9	0.87	9.4
2-3-1	3.6	0.87	9.4
3-0-0	2.3	0.46	9.4
3-0-1	3.8	0.87	11.
3-0-2	6.4	1.7	18.
3-1-0	4.3	0.90	18.
3-1-1	7.5	1.7	20.
3-1-2	12.	3.7	42.
3-1-3	16.	4.0	42.
3-2-0	9.3	1.8	42.
3-2-1	15.	3.7	42.
3-2-2	21.	4.0	43.
3-2-3	29.	9.0	100.
3-3-0	24.	4.2	100.
3-3-1	46.	9.0	200.
3-3-2	110.	18.	410.
3-3-3	>110.	42.	---

Note 1: FC can also be tested by the ‘5-replicate tube method’, where five replicate tubes per dilution are used instead of three replicate tubes as described in the procedure here. Except MPN calculation, the rest of the procedure remains same for both the methods. Procedure and tables given in Annexure 1 should be followed for calculation of MPN using the 5-replicate tube method.

Note 2: For the 5-replicate tube method, $MPN / 100 \text{ mL} = MPN \text{ from chart in Annexure 1} \times (\text{ml sample for first column of chart} / \text{ml sample in first dilution of the selected series}) \times 100$

5.18 ENUMERATION OF MALE-SPECIFIC (F+) COLIPHAGE (MS2 COLIPHAGE) IN WASTEWATER AND FAECAL SLUDGE

1. Scope and objective

The adoption of this method describes the detection of male-specific (F+) coliphage (MS2 coliphage) in the effluent, faecal sludge and dried or composted sludge.

2. Principle

Coliphages are viruses (bacteriophages) that infect *E. coli* and are indicators of faecal contamination. This method is capable of detecting male-specific (F+) coliphages. MS2 coliphage is non-pathogenic to humans, it is surrogates for enteric viruses and suitable to analyse in the laboratory. F-factor is the fertility factor in certain strains of *E. coli*. It is a plasmid that when present codes for pilus formation. The pilus allows for the transfer of nucleic acid from one bacterium to another. Male-specific coliphage (F+) are RNA or DNA viruses that infect via the F-pilus of the male strain of *E. coli*.

Coliphage presence in ground water is an indicator of faecal contamination. Method 1601 (two step enrichment procedure) is a performance-based method for detecting the presence of male- specific (F+) and somatic coliphage in the ground and other water. This method is intended to help determine if ground water is affected by faecal contamination.

3. Apparatus

- 50 ml centrifuge tubes for collection of samples;
- 1.5 ml or 2 ml sterile microcentrifuge tubes with racks;
- 10 ml sterile test tubes with rack;
- 500 ml and 100 ml glass (duran) bottles;
- 15 x 90 mm plastic sterile petri dishes;
- 125 ml or 250 ml erlenmeyer flasks;
- Plastic pasteur pipettes (plastic dropper);
- 1–10 ml auto pipette and sterile tips;
- 10–100 µl auto pipette and sterile tips;
- 100–1000 µl auto pipette and sterile tips;
- 5 ml or 15 ml sterile syringes;
- 0.45 µm and 0.2 µm syringe filters;
- Mortar and pestle;
- Inoculation loop;
- Parafilm;
- Bunsen burner;
- Incubator;
- Shaker incubator;
- Spectrophotometer and cuvettes;
- Laminar air flow;
- Weighing balance;
- Vortex mixer;
- Microcentrifuge;

-
- Hot water bath;
 - Autoclave.

4. Chemical and reagents

1. Reagent water (phosphate buffer solution, PBS)

- Dissolve 1 PBS tablet in 1 L of distilled water. Sterilize in an autoclave at 121°C and 15 psi for 15 minutes.
- Store at 4 °C ± 1 °C for < 3 months.

2. Conc. glycerol (>99.5%: molecular weight 92.09)

- For collection of *E. coli* F_{amp} from broth in freezer -20 °C.
- Autoclave for 15 minutes at 121 °C and 15 psi. Remove promptly to avoid scorching. Store at room temperature. It should not be prepared too much and should be used only once.
- Store at 4°C ± 1°C.

3. 70% Glycerol (molecular weight 92.09)

- For collection of *E. coli* F_{amp} from agar in freezer -20°C.
- Dissolve 70 ml of conc. glycerol with 30 ml of distilled water. Autoclave for 15 minutes at 121°C and 15 psi. Remove promptly to avoid scorching.
- Store at 4°C ± 1°C until use.

4. Antibiotic stock

Stock ampicillin/streptomycin

- For growth of *E. coli* F_{amp}, the host bacteria for male-specific coliphage.
- Dissolve 0.15 g of ampicillin sodium salt (Sigma A9518) and 0.15 g streptomycin sulphate (Sigma 56501) in 100 ml of reagent water. Filter through a sterile 0.20 - 0.22 µm pore-size membrane filter assembly. Dispense 1 ml per 2 ml microcentrifuge tubes, label date on vial.
- Store frozen at -20 °C for one to two years. Thaw before use at room temperature or rapidly in a 36 °C ± 1.0 °C water bath. Mix well before use.

5. Tryptic (or trypticase) soy broth (TSB)

- For growth of *E. coli* F_{amp} the host bacteria for male-specific coliphage.
- Follow procedure as specified on the bottle of media. After melting and mixing, dispense 25 ml of TSB into 125 ml or 250 ml of Erlenmeyer flasks and make a cork with cotton wool and gauze. (Adjust pH to 7.3 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary). Autoclave at 121 °C and 15 psi

for 15 minutes (check pH again after autoclaving by using representative flask. Adjust pH as necessary).

- Store at $4\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for < 1 month.

6. TSB with streptomycin/ampicillin

- For growth of *E. coli* F_{amp}, the host bacteria for male-specific coli phage.
- Add 250 µl of stock streptomycin/ampicillin sulphate to 25 ml of autoclaved TSB in flask. *Please note: Antibiotics must always be added to the medium after the medium has been autoclaved and cooled.*

7. 1.5 % Tryptic soy agar (TSA)

- For growth of *E. coli* F_{amp} in streak plates.
- Follow procedure as specified on bottle of media. Add 15 g of agar per 1 L of TSB (adjust pH to 7.3 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary). Autoclave for 15 minutes at 121°C and 15 psi. Cool to $48\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$ and mix molten medium well and pour in to the petri plates. Store inverted at $4\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for < 3 months.
- Add 10 ml stock ampicillin/ streptomycin sulfate to 1 L of autoclaved 1.5 % TSA. *Please note: Antibiotics must always be added to the medium after the medium has been autoclaved and cooled.* Swirl flask until well mixed and aseptically dispense 17–18 ml per 90 mm plastic sterile petri dishes. Allow to solidify with lids off in a biohazard hood for several minutes prior to use.
- If not used immediately, replace lids and store inverted at $4^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for less than two weeks.

8. 0.7 % Tryptic soy agar (TSA)

- ‘Soft’ agar for enumeration of MS2 Coliphage.
- Follow procedure as specified on bottle of media. Add 7 g of agar per 1 L of TSB (adjust pH to 7.3 with 1.0 N hydrochloric acid or 1.0 N sodium hydroxide, if necessary). Autoclave for 15 minutes at 121 °C and 15 psi. Cool to $48^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and mix molten medium well and pour in to the Petri plates. Store inverted at $4^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for < 3 months.
- Add 10 ml stock ampicillin/streptomycin sulfate to 1 L of autoclaved 0.7% TSA. *Please note: Antibiotics must always be added to medium after the medium has been autoclaved and cooled.* Dispense 5 ml per sterile 10 ml test tube, and label date. Keep at 45°C to 48°C until use.
- Tubes must be used the day they are prepared.

Reference strains used

1. *E. coli* F_{amp} ATCC # 700891-reference strain freeze dry.

E. coli F_{amp}- *E. coli* HS (pFamp)R (male-specific coliphage host)-originated by Victor Cabelli, formerly of the Department of Microbiology, University of Rhode Island, Kingston, RI, USA, frozen stock. ATCC#700891. May be stored at 2°C to 8 °C for < 5 years.

- Generation 1st- in 1.5% TSA with antibiotic: Sterilize the inoculating loop in the Bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool. Strike slightly the reference strain powder (in form freeze dry) and streak it over the first quadrant (approximately 1/4 of the plate) on 1.5% TSA with antibiotic using streak plate technique. Incubate at 37°C for 24 hours.

Harvest agar by cutting the agar within the area of colonies grown into pieces and put four to five agar pieces in 1.5 ml or 2 ml microcentrifuge tubes. Add 70 per cent glycerol to almost full of tubes. If stored at -20 °C, the host bacteria may be retained for less than year (i.e. approximately seven to eight months).

First generation in TSB with antibiotic: Sterile the inoculating loop in the Bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool. Strike slightly the reference strain powder (in form freeze dry) and put it into 25 ml of TSB with antibiotic in flask. Incubate in shaking incubator at 37°C, 150 rpm for 20–24 hours.

Harvest broth by mixing sterile glycerol and broth with the host bacteria in a ratio of 1:4 in 1.5 ml or 2 ml microcentrifuge tubes. Add 200 µL sterile glycerol by plastic dropper plus 800 µL *E. coli* F_{amp}. Label with *E. coli* strain and date of harvest. Host bacteria stored at -70 °C may be retained for up to one year. If stored at -20 °C, the host bacteria may be retained for less than one year (i.e. approximately seven to eight months).

- Log-phage *E. coli* F_{amp}, the host bacteria for male-specific coliphage.

Second generation (working strain) in TSB with antibiotic: Add 100 µL of stock *E. coli* host first generation into 25 ml of TSB with antibiotic in flask. Incubate in shaking incubator at 37°C and 150 rpm for two hours (OD = 0.4–0.5) or until cultures are visibly turbid (cloudy), indicating log-phase growth. *Please note: Aseptically remove 2–3 ml of culture from the flask, dispense into a cuvette and*

read absorbance at 520 nm. An absorbance reading of 0.1–0.5 optical density (OD) units is an indication of log-phase growth. If proper OD has not been reached, place cultures back into shaker incubator and take readings every 30 minutes until an OD of 0.1–0.5 is reached.

2. MS2 stock coliphage ATCC#15597-B1-Reference strain freeze dry

MS2 stock coliphage (ATCC#15597-B1): Male-specific (F+) coliphage for initial preparation of stock. May be stored at 2–8°C for less than five years.

- Working strain for positive control in autoclaved PBS: Sterilize the inoculating loop in the Bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool. Strike slightly the reference strain powder (in form freeze dry) and put it into 9 ml of PBS in 10 ml test tube as Dilution factor 0. Make dilution series until -8 to -20 in 1.5 microcentrifuge. Choose dilution factor between -8 to -20 to make positive control. For the remaining MS2 in PBS solution, it can be used for creating a next series dilution.
- For freeze dry, store at 2–8°C for less than five years.
- For in solution form (PBS), store at -20°C less than or equal to one year.

5. Procedure

Sample collection, preservation, storage and preparation

- Collection and transportation: Samples are collected in sterile plastic bottles or 50 ml centrifuge tubes and shipped to the laboratory for analysis. Samples must be shipped at 2–8 °C using wet ice or similar products to maintain temperature. The sampling team must maintain a log book with the following information for each sample: name, sample location, date and time of collection.
- Storage: Samples must be stored at 4°C ± 1°C. Do not freeze.
- Sample size:
 - o using 0.5 ml of sample: Collect 50 ml of sample for each of sample type.
 - o using 1 g of sample: Collect 25 g of sample for each of sample type.
- Holding time: The following are maximum holding times beyond which the sample cannot be retained for testing or the results are low accurate. Raw sewage sample is analysed between collection of sewage sample and analysis in 24 hours.
- Dechlorination procedure: Although this method was validated for use with unchlorinated groundwater, it potentially can be used with chlorinated waters.

If the sample has been chlorinated, add 0.025ml (25 µL) of 10 per cent sodium thiosulphate per 50 ml of sample at time of sample collection.

- Sample preparation—liquid sample: Homogenize sample by vortex at top speed for two to three minutes and filter 2 ml of sample through 0.45 µm sterile syringe filter into sterile 2 ml micro centrifuge tubes. Make dilution series with PBS solution.
- Solid sample: Randomly weight 1 g into a 10 ml test tube with 9 ml of sterile PBS. Homogenize sample by vortex at top speed for two to three minutes. Make dilution series with PBS solution.

Procedure for enumeration of male-specific (F+) coliphage (MS2 Coliphage)

Positive control

- Prepare MS2 Coliphage by following the procedure given in 'Working strain for positive control' in 'MS2 stock coliphage ATCC#15597-81-Reference strain freeze dry'.
- Add 100 µL of log-phase *E. coli* host and 500 µl of MS2 Coliphage in PBS to sterile test tubes.
- Place the test tubes into water bath (47 °C) and add 6 ml of 0.7% TSA with antibiotic (0.7% TSA).
- Take one by one, mix tube in palm and then pour on plates and allow to solidify and incubate 24 h at 37 °C.
- Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 24 h of incubation are plaques. Report as presence/ absence for positive control

Negative control

- Add 100 µl of log-phage *E. coli* host and 500 µl of sterile PBS to sterile test tubes.
- Place the test tubes into water bath (47°C) and add 6 ml of 0.7% TSA with antibiotic
- Take one by one, mix tube in palm and then pour on plates and allow to solidify and incubate for 24 hours at 37°C.
- Circular zones of clearing (typically 1n to 10 mm in diameter) in lawn of host bacteria after 24 hours of incubation are plaques. Report as presence/absence for positive control

Sample

- Prepare sample by following sample preparation as described above.
- Add 100 µL of log-phage *E. coli* host and 500 µL of sample in PBS to sterile test tubes for all dilution factors (two replicates per dilution).

- Place the test tubes into a water bath (47°C) and add 6 ml of 0.7 per cent TSA with antibiotic.
- Take one by one, mix tube in palm and then pour on plates and allow to solidify and incubate 24 hours at 37°C.
- Circular zones of clearing (typically 1–10 mm in diameter) in lawn of host bacteria after 24 h of incubation are plaques. Count the number of plaques on each plate and calculate the PFU/ml.

6. Calculation

The number of plaque-forming units (PFU) per ml in the coliphage spiking suspension will be calculated using DAL plates that yield plaque counts within the desired range of zero to 300 PFU per plate for male-specific (F+) coliphage and zero to 100 PFU per plate for somatic coliphage. There may be occasions when the total number of plaques on a plate will be above the ideal range. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as ‘too numerous to count’ (TNTC). For each coliphage type, sum the number of PFU from all dilutions with plaques (on either of the duplicate plates), excluding dilutions with all TNTC or all zeros. Add the undiluted sample volumes used to inoculate all replicate plates at all dilutions with useable counts (as defined above). Divide the sum of the PFU by the sum of the undiluted sample volume to obtain PFU/ ml in the spiking suspension.

The equation is as follows:

$$\text{PFU/ml} = (\text{PFU}_{\text{DF}^1} + \text{PFU}_{\text{DF}^2} + \dots + \text{PFU}_{\text{DF}^n}) / (V_1 + V_2 + \dots + V_n)$$

PFU = number of plaque forming units from plates of all countable sample dilutions (dilutions with 1 or more PFU per plate, excluding dilutions with all TNTC or all zeros)

DF = Dilution factor

V = Volume of diluted sample in all plates with countable plaques.

N = Number of useable counts

Chapter 6

6.0 Analysis of biosolids

6.1 STANDARD OPERATING PROCEDURE FOR ANALYSIS OF pH IN BIOSOLID

Material requirements

- 250 ml beaker
- Analytic balance
- Filter paper
- Tissue roll
- pH meter

Procedure

- Take 20 g of powdered biosolid and prepare suspension in 100 ml of distilled water in order to maintain the ratio of 1:5 and shake on a rotary shaker for 2 hours.
- Allow the suspension to settle for three to four hours.
- Filter through Whatman No. 1 or equivalent filter paper under vacuum using a Buchner funnel.
- Determine pH of the filtrate by pH meter as mentioned in the chapter 5, section 5.1

6.2 STANDARD OPERATING PROCEDURE FOR ANALYSIS OF ELECTRICAL CONDUCTIVITY IN BIOSOLID

Material requirements

- 250 ml flask
- 100 ml beaker

Instrument

- Analytic balance
- Conductivity meter [Cyberscan 200]

Procedure

- Grind the sample using mortar and pestle
- Sieve the sample through a 2–4 mm sieve.
- Take 20 g of the sample and add 100 ml of distilled water to it obtain a ratio of 1:5.
- Stir for about an hour at regular intervals.
- Allow the suspension to settle for 3–4 hours.
- Immerse the probe of conductivity meter into the suspension and measure the conductivity of the unfiltered biosolid suspension

Calculation

The results will be displayed as $\mu\text{S}/\text{cm}$ or mS/cm at 25°C in the display of conductivity meter. However, the results are reported as dS/m by using the following mentioned conversion factor.

$$1 \text{ mS}/\text{cm} = 1 \text{ dS}/\text{m}$$

$$1000 \mu\text{S}/\text{cm} = 1 \text{ dS}/\text{m}$$

Note: While reporting the conductivity, specify the dilution of the bio solid suspension viz., 1:5 biosolid suspensions.

6.3 STANDARD OPERATING PROCEDURE FOR ANALYSIS OF MOISTURE CONTENT IN BIOSOLID

Material required

- Petri dish
- Weighing balance
- Oven
- Desiccator
- Butter paper

Procedure

- Take the weight of an empty clean petri dish or aluminium evaporating dish (W_1) and tare it.
- Add 10–30 g of the sample to be tested into the petri dish or aluminium evaporating dish and note down the weight (W_2).
- Dry the petri dish or aluminium evaporating dish along with the sample overnight in hot-air oven at $103\text{--}105^{\circ}\text{C}$.
- After drying, keep the sample in a desiccator and allow it to cool. Take the weight of the petri dish with the dried sample (W_3).

-
- Subtract the empty petri dish (W1) from the weight of the petri dish with the dried sample (W3) to get the weight of the dried sample alone.
 - Calculate the moisture percentage as per the formula given below.

Note: The drying oven should be kept in a hood or be vented. Significant laboratory contamination may result from drying a heavily contaminated sample.

Calculation

$$\text{Moisture \%} = \frac{W_2 - (W_3 - W_1)}{W_2} \times 100$$

W₂

- W1 = Weight of empty petri dish.
- W2 = Weight of wet sample in tared petri dish
- W3 = Weight of petri dish with dry sample

6.4 STANDARD OPERATING PROCEDURE FOR CARBON, HYDROGEN, NITROGEN ANALYSIS BY CHN ANALYSER (LECO MODEL-828 SERIES)

Scope and application

The elemental analyser is a device used for quantitative determination of basic elements carbon, hydrogen, nitrogen, and sulphur in organic samples. A wide range of solid or liquid organic samples from various industries such as environment, geology, food, chemical, and pharmaceutical can be examined. In the environmental sector, carbon, hydrogen, and nitrogen content of any biomass, compost, organic manure, biosolids, and soil can be analysed.

Principle

CHN Analyser works on the principle of Dumas method, which is based on the combustion of a material at or near 1000°C in the presence of oxygen. In the combustion process (furnace at 1000°C), carbon is converted to carbon dioxide (CO₂); hydrogen to water (H₂O); nitrogen to nitrogen gas/ oxides of nitrogen (N₂/NO₂). The combustion products are swept out of the combustion chamber by an inert carrier gas such as helium and passed over heated (about 600°C) high-purity copper. The copper removes any oxygen not consumed in the initial combustion and converts any oxides of nitrogen to nitrogen gas. Combustion gases are swept from the furnace through an afterburner furnace containing reagent to scrub sulfur compounds from the gas stream before collection in the ballast volume. The gases equilibrate and mix within the ballast before a representative aliquot of the gas is extracted and introduced into a flowing stream of inert gas for analysis. The

aliquot gas is carried to a non-dispersive infrared (NDIR) cell for the detection of carbon (as carbon dioxide) and a thermal conductivity cell (TC) to detect nitrogen (N_2). The gas is also transferred to the H_2O NDIR cell for the determination of hydrogen. An external PC with LECO Cornerstone brand software manages all the quantitative calculations and saves all the data.

Equipment

CHN Analyser (LECO, Model-828 series)

Chemicals and consumables

Sample holder, forceps, tin foil, three gas supplies:

- (i) an inert carrier gas (helium);
- (ii) high purity oxygen (minimum 99.9995%); and
- (iii) zero air

Calibration standard (LECO certified reference material)

Part no.	Description	% C	%H	%N
502-896	EDTA LCRM	41.00±0.15	5.52±0.06	9.57±0.04

Sample preparation

Biosolid samples are oven-dried for 24 hours at 105°C to remove the moisture content completely. To homogenize the sample, it should be grinded using mortar and pestle before analysis.

Procedure for CHN Analysis

- Switch 'On' the instrument - LECO, Model-828 series.
- Switch 'On' the system connected to the instrument.
- Click On the software named 'Cornerstone'.
- Turn the gas cylinder valve of helium, zero air, and oxygen to 'On' position.
- Set the pressure according to the mentioned limits (Helium:25 psi; zero air:14 psi; oxygen: 25 psi).
- In the software, 'go to the diagnostics' window and click ambient which displays all the parameters.
- Set the 'gas on' in the software.
- Wait till the furnace temperature rises to 950°C, afterburner temperature to 850°C and ballast oven temperature to 45°C.
- Wait till the thermal conductivity (TC) cell signal becomes stable.
- In the meanwhile, weigh around 0.1 g of standard in tin foil.
- When all the instrument parameters have reached the required range and value, click the 'system check' tab in the diagnostics window.

-
- Once the system check is completed without any failure, the icon 'ready to analyse' will be automatically enabled.
 - Initially, analyse CHN for the standard reference material provided by the manufacturer to check the proper functioning of the instrument.
 - Go to the analysis window, fill in the details of the standard reference material used.
 - Once the analysis is completed for the standard, check the determined values are in accordance with the values provided for the standard reference material.
 - If No, then check for the leakage of gas or any other malfunctioning elements associated with the instrument.
 - Rectify the issue and again run the standard until the required values are achieved.
 - If yes, then proceed with sample analysis.
 - Fill in the sample details and load the samples according to the loader location which appears in the analysis window.
 - Once all the samples are loaded, click 'ready to analyse' (maximum of 30 samples can be loaded at a time).

Result interpretation

- The calculation for the conversion of CO₂, H₂O and NO₂ to the percentage of carbon, hydrogen and nitrogen is done by the software.
- The results are displayed in the form of graphs. The determined values can be expressed in %, mg/Kg, or ppm.

6.5 STANDARD OPERATING PROCEDURE FOR SULPHUR ANALYSIS BY SULPHUR ANALYSER (LECO MODEL-832 SERIES)

Scope and application

A sulphur analyser is an instrument that is used for the quantitative determination of sulphur in any organic or inorganic samples. Sulphur content in food products, drugs, fertilizers, organic manure, plastics, biomass, agricultural residue can be determined. In the sanitation sector particularly, the instrument is helpful for determining the sulphur content of sewage sludge, and dried faecal sludge (biosolid).

Principle

Sulphur analyser works on the principle of the Dumas method, which is based on the combustion of a material at or near 1000°C in the presence of oxygen. During the combustion process, sulphur is converted to sulphur dioxide (SO₂).

Analysis begins as a sample is weighed into a combustion boat (unglazed ceramic) and placed into the furnace typically regulated at 1350°C with a pure oxygen environment. The sample combusts, releasing carbon as CO₂ gas with the sulphur forms being oxidized and released as SO₂ gas. The combustion gases flow through anhydron tubes removing moisture and to the flow controller, setting the flow of the combustion gases through the NDIR sulfur detection cells.

Chemicals and consumables

Unglazed ceramic crucible for holding sample and oxygen supply (purity required: minimum 99.9995%)

Calibration standard (LECO certified reference material)

Part No.	Description	%S
502-897	BBOT LCRM	7.46±0.07

Equipment

Sulphur analyser (LECO, Model-832 series)

Sample preparation

Biosolid samples are oven-dried for 24 hours at 105°C to remove the moisture content completely. To homogenize the sample, it should be grinded using mortar and pestle before analysis.

Procedure

- Switch 'On' the instrument: LECO, Model-832 series
- Double click the software named 'Cornerstone'
- Turn the gas cylinder valve of oxygen to 'On' Position
- Set the pressure according to the mentioned limits (Oxygen: 15 psi)
- In the software, go to the diagnostics window and click ambient which displays all the parameters
- Set the 'gas on' in the software.
- Wait till the furnace temperature rises to 1350°C.
- When all the parameters have reached the required range and value, click the 'System check' tab in the diagnostics window.
- In the meanwhile, weigh around 0.1 g of reference standard and place it in an unglazed ceramic crucible.
- Once the system check is completed without any failure, the icon 'Ready to analyse' will be automatically enabled.
- Go to analysis window, fill in the sample details including sample ID, sample weight etc.

-
- Click 'Ready to Analyse'. After the stabilization of baseline, wait for the software commands for 'Sample loading'.
 - Load the sample in the horizontal furnace and click the icon 'Analyse'.
 - Once the analysis is completed for the standard, check the determined values are in accordance with the values provided for standard reference material.
 - If No, then check for any leakage of gas or any other malfunctioning elements associated with the instrument.
 - Rectify the issue and again run the standard until the required values are achieved.
 - If yes, then proceed with sample analysis.
 - Take the crucible out of the chamber and wait for the software command to load the next sample.
 - Weigh around 0.3 g of sample and place it in an unglazed ceramic crucible and repeat the same procedure mentioned for standard for sample analysis.

Result interpretation

- The calculation for the conversion of SO₂ to the percentage of sulphur is done by the software.
- The results are displayed in the form of graphs. The determined values can be expressed in per cent (%), mg/Kg, or ppm
- To save the results, select the data for which results are to be saved, then go to output, click print, and save in the required folder

6.6 STANDARD OPERATING PROCEDURE FOR CALORIFIC VALUE ESTIMATION BY BOMB CALORIMETER (LECO AC 500)

Scope and application

Bomb calorimeter is used to determine the calorific value of solid and liquid fuels. The energy content in kerosene, petrol, wood chips, cow dung, agricultural biomass can be determined. Biosolids contain energy content similar to other agricultural biomasses which are currently used as solid fuel. The potential of biosolids or other bio-based materials to replace non-renewable sources of energy can be determined based on their calorific value.

Principle

The bomb calorimeter consists of pressurized oxygen 'bomb' (30 bar), which houses the fuel. At high oxygen pressure complete combustion of sample occurs if the sample contains energy content.

Equipment

Bomb calorimeter (LECO AC 500)

Chemicals and consumables

- Nichrome wire (10 cm),
- Oxygen supply (99.995%)

Calibration standard (LECO certified reference material)

Part no.	Description	Heat of combustion
502-892	Benzoic acid LCRM	6315.6±9.4 cal/g

Sample preparation

Biosolid samples are oven-dried for 24 hours at 105°C to remove the moisture content completely

Procedure

- Switch 'On' the instrument.
- Switch 'On' the system connected to the bomb calorimeter.
- Double Click the software named 'AC 500'.
- Click the 'Water pump' on to start the circulation of water throughout the instrument.
- Calibrate the instrument by running the reference standard material provided by the manufacturer.
- Benzoic acid is the standard material provided along with LECO 500.
- Weigh and place the standard in the crucible.
- Prepare the combustion vessel by connecting a 10 cm fuse wire between two electrodes and keep close to standard material taken in the crucible inside the bomb.
- Close the combustion vessel and fill in oxygen until it reaches 28.96 bar.
- After filling the oxygen, the combustion vessel is placed in a container filled with 2 L of deionized water.
- Place the container containing the vessel inside the instrument and close the circuit of electrodes.
- In the software, mention the details of the standard such as name and weight and then click on the 'Analyse' button.
- The temperature of the water is measured by means of a precision thermocouple.
- A stirrer stirs the water continuously.
- Initially, the temperature change would be small as the only heat generated would be from the stirring of the water molecules. This is the time taken for equilibration. It takes three minutes for equilibration process.

-
- After the temperature is stabilized, the sample is fired, i.e a high voltage is sent across the electrodes and through the fuse wire.
 - The electric current passing through the fuse wire would almost instantly ignite and combust the fuel sample in the presence of oxygen.
 - The water absorbs the heat, released by the combustion of the sample, resulting in a sharp rise in the water temperature. The temperature continues to rise for some time before levelling off
 - The water temperature is continuously recorded till the temperature readings are stable.
 - Sample analysis takes five minutes' time.
 - After completion of the analysis, disconnect the electrodes and release the oxygen pressure
 - When all the pressure is released, open the combustion vessel.
 - Wipe the water molecules formed inside the combustion vessel and prepare the next sample for analysis.
 - After sample preparation, place approximately 1 g of sample in the crucible and carry out the procedure as mentioned above for the standard.

Result interpretation

- The gross calorific value of the sample is calculated based on the initial mass of the fuel sample and the change in temperature by the software.
- The results are displayed as 'Cal/g' in the analysis window.

6.7 STANDARD OPERATING PROCEDURE FOR ESTIMATION OF HEAVY METALS IN BIOSOLIDS AND WASTEWATER SAMPLE BY ICP-OES (PERKIN ELMER; AVIO 200)

Scope and application: This procedure is applicable for the estimation of heavy metals in biosolids and faecal sludge sample by ICP-OES

Atomic emission spectroscopy (AES), more commonly known as optical emission spectroscopy (OES). It relies on the fact that excited atoms emit electromagnetic radiation.

Inductively Coupled Plasma (ICP) Optical Emission Spectroscopy (OES) is a sensitive and widely used analytical technique used to determine the elemental composition of a broad range of metals. It is an extremely time-efficient and accurate method for determining the constituents of a variety of metals and alloys. It is done using an electromagnetically charged apparatus, which utilizes gas flow

techniques to collide nebulized liquid samples with extremely hot plasma. The method also uses mass spectroscopy equipment to analyse the ionization of gas particles that flow from the plasma channel.

The ICP is primarily used in the process of atomic determination of trace elements in liquid samples. Ionized particles are measured in an optical sample chamber with the help of the incident light of the plasma torch and precise optical sensors. Each element is categorized by its specific wavelength and light intensity characteristics.

In a typical ICP spectroscopic array, liquid samples are pneumatically nebulized and passed on to argon plasma via a cold flow of gas. After that, they are vaporized in the argon plasma and subsequently ionized. Large particles are unsuitable for ICP analysis. Therefore, the larger liquid particles are separated from the analyte cross flow in a spray chamber. It is hard to convey this methodology in the ICP analysis of solid samples. For that reason, when analysing solid samples, they must be first liquefied through dissolution, which is done by acid digestion.

ICP OES takes on this role, superseding other methods of elemental analysis. Short analysis times, low sample preparation effort, multi-element detection and exceptional device sensitivity are hallmarks of this method. The latest generation of ICP OES devices makes elemental analysis even more user-friendly, economical, and flexible.

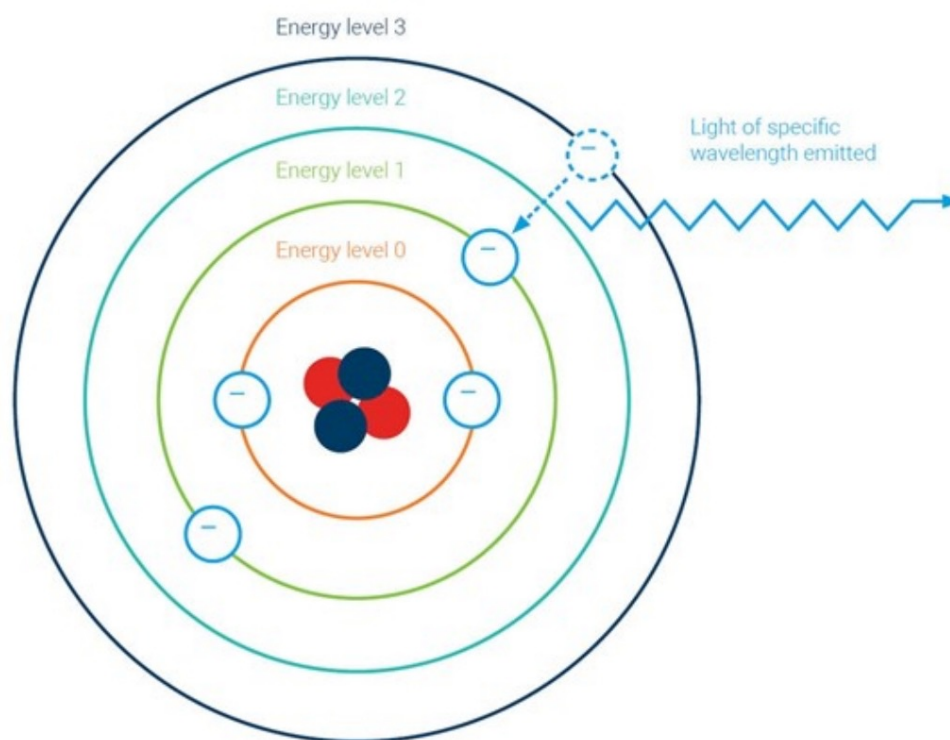
Principle

Inductively Coupled Plasma Optical Emission spectroscopy/atomic emission spectroscopy (ICP-OES/ AES) is an analytical technique used to determine how much of certain elements are in a sample. The ICP-OES principle uses the fact that atoms and ions can absorb energy to move electrons from the ground state to an excited state. In ICP-OES, the energy in the form of heat generated from an argon plasma that operates at 10,000 Kelvin.

The ICP-OES principle exploits the fact that the excited atoms release light of a specific wavelength as they transition to lower energy state. The amount of light released at each wavelength is proportional to the number of atoms or ions making the transition. The Beer Lambert law describes the relationship between light intensity and concentration of the element.

As an electron returns from a higher energy level to a lower energy level, usually the ground state, it emits light of a very specific wavelength. The type of atom or ion (i.e. which element it is), and the energy levels the electron is moving between, determines the wavelength of the emitted light.

Diagram 1: Energy levels in a lead (Pb) atom



Courtesy: Agilent Technologies

Diagram 1 shows the energy levels in a lead (Pb) atom. Energy from the heat of the plasma causes electrons to become 'excited' and move to higher energy levels. The amount of energy required for the electrons to move between levels corresponds to very specific wavelengths of light. As the diagram shows, moving an electron from the ground state to the first energy level (E1) requires energy equivalent to light at 283.3 nm. The 283.3 nm wavelength is in the ultraviolet (UV) part of the electromagnetic spectrum.

It takes more energy to move an electron from the ground state to the second energy level (which is further away from the nucleus). This transition requires more energy, equivalent to a photon of light at 261.4 nm, which is further into the UV spectrum. As the diagram shows, lead will emit light at 283.3, 261.4, 217.0 and 202.2 nm, depending on which energy level the electron is returning from.

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) is a method of emission spectroscopy that excites atoms and ions with a plasma, causing

it to emit electromagnetic radiation at wavelengths characteristic of a particular element. Atoms absorb light of the same wavelengths that they themselves emit when luminescing. Because atoms can only absorb energy in specific quanta, the light they emit consists of different frequencies. Consequently, by looking at the spectrum of the absorbed or emitted light, it is possible to distinguish atoms clearly—two points of departure for elemental analysis.

ICP OES uses an inductively coupled plasma to excite the atoms in the sample. A generator supplies argon gas with enough energy for it to jump to the next physical state. With a high particle density, the plasma reaches temperatures of 5,000 to 10,000 K. The measurement solution is then injected into the plasma. It takes a fraction of a second for the sample to dry out, melt and finally to vaporize. The gas molecules, which are now also being excited, are then atomized and ionized. They emit the electromagnetic radiation that is used for the actual analysis. Transfer optics direct the radiation to another optical component that can separate out the various wavelengths. After the waves are split, a detector registers the intensity of each wavelength, which is proportional to the concentration of the respective analyte. As the analysis depends on the correlation between the light intensity and the element concentration, calibration standards come into play. These calibration standards help to derive a mathematical function which relates radiation to concentration.

Components of an ICP OES

The following are the technical components of an ICP OES device.

- **Plasma torch:** This component is responsible for maintaining the plasma. The torch also has an injector which introduces the previously atomized sample into the plasma from the upstream sample insertion system.
- **High-frequency generator:** The plasma is coupled to a high-frequency generator responsible for feeding energy to the plasma. It operates at frequencies of 27 or 40 MHz.
- **Sample insertion system:** Many components come together here. A peristaltic pump delivers the measurement solution to the atomizer. There, a gas stream disintegrates the fluid into droplets. The downstream spray chamber serves to remove larger droplets from the resulting aerosol. The injector in the torch is connected to this component.
- **Transfer optics:** The transfer optics are not responsible for separating the wavelengths. They only serve to transmit the radiation to the dispersing optics.
- **Monochromator/polychromator:** Separation into the component wavelengths can be accomplished in two ways: either sequentially (monochromator) or

simultaneously (polychromator). With ICP OES it is critical that the optics can distinguish adjacent lines that are extremely close to each other.

- Detector: A CID or CCD sensor is used to detect the signals. The incident light on the sensor induces a change in charge which is processed as a signal.

Equipment

ICP-OES (Perkin Elmer; AVIO 200)

Chemicals and consumables

All chemicals and reagents are high pure elemental analysis grade

- Nitric acid 70%
- Hydrochloric acid 37 %
- Hydrofluoric acid 51%
- Hydrogen peroxide 30 %
- Water (High pure)
- Potassium iodide
- Ascorbic acid
- Sodium borohydride
- Syringe (5mL)
- Syringe filter (0.45µm)
- Calibration standards/standard stock solution

Sample preparation

Biosolid samples are oven-dried for 24 hours at 105°C to remove the moisture content completely

Procedure for biosolids sample digestion in microwave digestion system (Milestone)

- Weigh 0.5gm to 1.0 gm of dried biosolids samples.
- Transfer it to microwave digestion vessels.
- Add 5 mL conc. nitric acid, 2 mL conc. HCl, 1 mL H₂O₂ and 1mL conc. hydrofluoric acid.
- Keep five minutes for open digestion.
- Add 2 mL high pure water.
- Close the vessels and keep them in microwave digester.
- After the digestion sample should cool down at room temperature.
- After cooling open the vessels, transfer the digested samples to a 50 mL centrifuge tube and rinse the vessels once or twice with 10 mL of water and finally make up to 50 mL with water/desired dilution.
- Filter the samples with a syringe filter (0.45µm).

- Use these filtered samples for ICP-OES analysis.

Note: All activity should be carried out in the fume hood.

Procedure for wastewater sample preparation

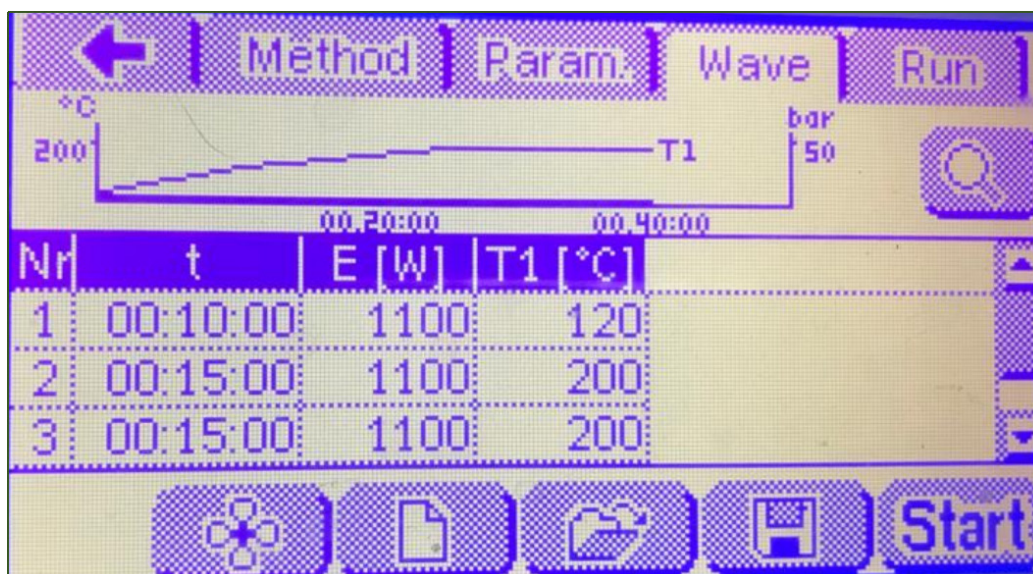
If wastewater does not have any sediment or particles;

- Take 5–10 mL samples and filter through a syringe filter (0.45µm).
- Add 20 mL of 10% HNO₃ desired dilution and mixed.
- Use these samples for ICP-OES analysis.

If wastewater has any sediment or particles follow the same procedure for biosolid sample preparation

- Close the vessels and keep them in microwave digester.
- After the digestion sample should cool down at room temperature.
- After cooling open the vessels and transfer the digested samples to a 50 mL centrifuge tube and rinse the vessels with once or twice with 10mL of water and finally make up to 50 mL with water/desired dilution.
- Filter the samples with a syringe filter (0.45µ).
- Use these filtered samples for ICP-OES analysis.

Diagram 2: Microwave digestion programme



Standard preparation

- Prepare the standards in 10% HNO₃ in the desired range but standards should be a minimum of 5.
- Either by serial dilution or direct from the stock solution.

Standard and sample preparation (arsenic)

Reagents to be prepared:

- Reductant solution: 0.2% (W/V) NaBH₄ in 0.05% (W/V) NaOH solution: Carefully dissolve 0.25 g sodium hydroxide flakes in MQ water and then add 1 g sodium tetrahydroborate to this solution and makeup to 500 ml. Prepare fresh. (This solution is required for mercury analysis.)
- KI solution: Dissolve 5 g KI and 5 g ascorbic acid in 100 ml MQ water. Prepare fresh daily.
- 100 ppb element Stock solution of Arsenic: Pipette out 1 ml of above 10 ppm As a solution and dilute to 100 ml in a volumetric flask. This will be 100 ppb As a stock solution.

Preparation of calibration standards and samples:

1. For calibration blank and standards: Pipette out 0, 0.25, 0.5, 1.5, and 2.5 ml of 100 ppb stock solution in four different 50 ml volumetric flasks. Add 5 ml of Conc. HCl (30%) and 5 ml of 5% KI solution to each flask. Allow to stand the mixture for 30–40 minutes.
2. Then make all flasks up to 50 ml mark. This will give 0, 0.5, 1, 3, and 5 ppb calibration standards for both mercury and arsenic. (Micropipette is best for low-level analysis to avoid contamination and accurate pipetting.)

For samples: Pipette out 5 ml of sample in 50 ml volumetric flask and follow the same procedure as above (In case of water or samples containing no matrix, instead of 5 ml, 25 ml volume can be taken for reduction).

Instrument operation

- Switch 'On' the instrument: PerkinElmer, Model-Avio 200.
- Switch On the computer connected to the instrument.
- Click On the software.
- Turn the gas cylinder valve of Argon and Nitrogen to 'On' position.
- Start the air compressor
- Set the pressure according to the mentioned limits (Argon: 120 psi; Nitrogen: 80 psi; Compressed air: 80 psi) p
- Start the chiller and set the temperature at 15°C.

- Click on plasma to ignite.
- Make the method as per requirements, put the dilution factor in the sample details window and every sample should be analysed in three replicates.
- Load the samples in the autosampler.
- Once all the samples are loaded, click ready to analyse (maximum of 100 samples can be loaded at a time).

Result interpretation

- The results are displayed in the form of a spectrogram. The determined values can be expressed in %, mg/Kg, or ppm.
- RSD of 3 replicates of each sample should be within R5%.
- The correlation coefficient value of the calibration curve should be $r \geq 0.995$.
- For sample validation (precision and accuracy): once a year spiked samples should be processed and analysed as per the sample digestion procedure and analyse along with calibration curve standards or as and when required.

6.8 STANDARD OPERATING PROCEDURE FOR TESTING OF FAECAL COLIFORM AND *E.coli* IN BIOSOLIDS DERIVED FROM FAECAL SLUDGE BY MOST PROBABLE NUMBER (MPN) TECHNIQUE

1. Scope and application

Coliforms are a group of bacteria that are predominantly found in the gastrointestinal tract of warm-blooded animals including humans. They are also present in soils, surface waters and on plant surfaces. Specific genera included in this group are *Escherichia*, *Klebsiella* and *Enterobacter*. These bacteria are facultatively anaerobic, gram-negative, non-spore forming, rod shaped bacteria that are able to ferment lactose and produce acid and gas within 24 hours at 35°C. Faecal coliforms (FC) (thermotolerant coliforms) are specific group of coliform bacteria that have the ability to grow, ferment lactose and produce acid and gas at elevated temperatures. These FC bacteria are exclusively found in the intestines and faeces of humans and other warm-blooded mammals and hence their presence indicates faecal contamination. The predominant FC is *Escherichia coli*. *E. coli* is a typical FC which from ancient times has been used as an indicator organism to specifically indicate faecal contamination of various sources, including drinking water, surface water, groundwater and wastewater. Generally, it is expected that the density of these bacteria directly correlates with the probability of pathogen presence. The ease of cultivation and detection made these bacteria the standard indicator organisms in sanitation to indicate environmental/faecal contamination and thus indicating the potential presence of other pathogens. As these bacteria

(FC/*E. coli*) specifically indicate faecal contamination, their use as faecal indicators can be extended to faecal sources like FS, FS derived biosolids, FS liquid effluent etc. to estimate the densities of FC/*E. coli* present in these samples which gives an indication of the level of pathogen contamination present in these samples.

2. Principle

In this method, specific growth medium lauryl tryptose broth/lauryl sulphate broth (LTB/LSB) is used for the selective enrichment of coliforms in the initial step (presumptive test). LTB has lactose as a fermentable carbohydrate which is utilized by coliform bacteria including *E. coli* for growth at $35 \pm 0.5^\circ\text{C}$ and produce acid and gas within 24–48 hours of incubation. The gas production can be observed in the inverted Durham tube present in the fermentation tube which indicates a positive presumptive test for the presence of coliform bacteria. The positive presumptive tubes are later subjected to confirmatory test in specific confirmatory media, EC-MUG media, for confirming the presence of faecal coliform (FC) and *E. coli*. Gas production in EC-MUG medium within 24 h of incubation at $44.5 \pm 0.2^\circ\text{C}$ confirms the presence of FC. The fluorogenic substrate 4-methylumbelliferyl- β -D-glucuronide (MUG) is used in the EC-MUG medium as a specific substrate to detect *E. coli*. *E. coli* specifically possesses the enzyme β -glucuronidase which is capable of cleaving the MUG with corresponding release of the fluorogenic compound 4-methylumbelliferone when grown in EC-MUG medium at $44.5 \pm 0.2^\circ\text{C}$ within 24 ± 2 h or less. This fluorogenic compound under long wavelength UV radiation (366 nm) emits bright blue fluorescence which is a specific indication for the presence of *E. coli*.

3. Apparatus

- Latex gloves; conical flask, 2 L;
- Measuring cylinders, 1 L;
- Weighing balance; butter paper;
- Microwave oven/hot plate;
- Screw cap bottle, borosilicate glass, 500 ml;
- Culture media dispensing pump;
- Test tubes, rimless, borosilicate glass (18 × 150 mm, 15 × 150 mm);
- Durham tubes, borosilicate glass, 10 × 60 mm;
- Loose-fitting autoclavable plastic caps (18 mm, 15 mm diameter);
- Polygrid test tube stand, autoclavable (16 mm, 20 mm diameter);
- Test tube basket, autoclavable, 180 x 170 x 160 mm;
- Autoclave;
- Petri dish;
- Aluminium foil or crucible;

- Laboratory blender;
- Wide mouth bottle, polypropylene, autoclavable, 500 ml;
- Biosafety cabinet/laminar airflow;
- Bunsen burner or alcohol burner;
- Vortex mixer; micropipettes and tips;
- 3 or 3.5 mm diameter inoculation loop/sterile wooden applicator stick;
- Incubator/water bath;
- Long-wavelength (366 nm) ultraviolet (UV) lamp (6 W preferred).

4. Reagents

- Distilled water;
- Lauryl tryptose broth/lauryl sulphate broth (LTB/LSB) (HiMedia);
- EC-MUG media (HiMedia);
- 1 N HCl;
- 1 N NaOH.

5. Reagent preparation

5.1 Culture media

- Prepare required volumes of LTB and EC-MUG media.
- To prepare 1 L of LTB media, take 1 L of distilled water using a 1 L measuring cylinder, and add 500 mL into a 2 L conical flask. Weigh 35.6 g of dehydrated LTB media powder on a clean butter paper and add it into the 2 L conical flask containing 500 mL of distilled water and dissolve the media completely. Heat if necessary to dissolve. Add the remaining 500 mL of distilled water into the conical flask and mix to form a uniform solution.
- One litre of EC-MUG media is also prepared in a similar way as described above by taking 37.05 g of dehydrated EC-MUG media powder.
- Pour the media broth (LTB and EC-MUG separately) into the 500 mL screw cap bottle fitted with a culture media dispensing pump and dispense 10 ml portions of medium into 15 × 150 mm test tubes using the culture media dispensing pump.
- Insert a Durham tube in inverted position into each test tube containing media (LTB and EC-MUG) and make sure that the inner Durham tube is completely filled with media leaving no air spaces.
- Close the tubes with 15 mm autoclavable loose fitting plastic caps, place them in a test tube basket or test tube stand and sterilize by autoclaving at 121°C (15 PSI pressure) for 15 min.

5.2 *Water for serial dilution*

- Pour distilled water into the 500 mL screwcap bottle fitted with a culture media dispensing pump.
- Dispense 9 ml of distilled water into 18 mm x 150 mm tubes using the culture media dispensing pump.
- Close the tubes with 18 mm autoclavable loose fitting plastic caps, place them in a test tube basket or test tube stand and sterilize in an autoclave at 121°C temperature (15 PSI pressure) for 15 minutes.

6. Procedure

6.1 *Sample preparation/sample homogenization*

- Mix the sample thoroughly and weigh 30.0 ± 0.1 g in a sterile petri dish/ aluminium foil. If the sample has smaller solid materials like wooden chips etc., crush and grind them using mortar and pestle before weighing to obtain a homogeneous mixture. Larger and harder substances that are difficult to ground can be discarded.
- Take 270 mL of sterile distilled water in a sterile measuring cylinder.
- Add the sample into sterile laboratory blender jar.
- Add the 270 mL sterile distilled water into the blender jar, close the jar with lid and blend at high speed for 1–2 minutes. This is the ‘homogenized’ sample. Each mL of the homogenized sample contains 10^{-1} (0.1) g of the original sample. Adjust the pH to 7.0–7.5 using 1.0 N HCl or 1.0 N NaOH, if required.

6.2 *Sample dilution*

- The determination of faecal coliforms/*E. coli* by MPN method requires prior dilution of the samples. The extent of dilution depends on the type of sample being analysed. In the treated biosolid samples of faecal origin, it may be required to dilute the sample several folds (up to 10^{-6} or more) to accurately estimate the density of faecal coliforms/*E. coli*.
- To take adequate quantity of original sample for analysis, at the initial step of sample dilution, it is recommended to always add 10 mL of ‘homogenized sample’ to 90 mL of sterile dilution water and shake vigorously at least 25 times. This will dilute the original sample 10^{-2} times, i.e. each 1 mL aliquot from this step contains 10^{-2} [0.01] g of original sample. Label this dilution as ‘ 10^{-2} ’. Note: If 10 ml of sample is not taken for analysis, then proceed as follows: Take the ‘homogenized sample’ bottle and shake it vigorously several (25-30) times. Take 1 mL aliquot from this bottle and add to tube containing 99ml 0.2 mL sterile dilution water and vortex. This tube contains 10^{-2} g of original sample. Label this dilution as ‘ 10^{-2} ’.

- Take 1 ml aliquot from the tube with ' 10^{-2} g' of original sample and add to sterile tube containing 9 ± 0.2 mL sterile dilution water and mix by vortexing. This tube contains ' 10^{-3} g' of original sample. Label this dilution as ' 10^{-3} '.
- Depending on requirement, serially dilute the sample and label the dilutions as ' 10^{-4} ', ' 10^{-5} ', ' 10^{-6} ', ' 10^{-7} ' and so on.
- Do not suspend sample in dilution water for more than 30 min at room temperature to avoid death or multiplication of microorganisms.
- Use sterile pipette and tips at each step of dilution and inoculation to avoid cross contamination.

6.3 *Presumptive test*

- If media tubes are kept in a refrigerator after autoclaving, bring all tubes to room temperature (RT) before inoculation by keeping them outside at RT for 1-1.5 h.
- In the present procedure, three replicate tubes per dilution (sample volume/sample quantity) are used. Hence, this method is also referred as '3-replicate tube method'.
- Arrange tubes with LTB medium in rows of three tubes each in a test tube rack.
- Label media name, sample ID, sample dilution (10^{-1} , 10^{-2} , 10^{-3} and so on) and replicate number (10^{-1} .I, 10^{-1} .II, 10^{-1} .III, and 10^{-2} .I, 10^{-2} .II, 10^{-2} .III and so on) on each row of tubes before inoculation.
- Add 1 ml of sample from 10^{-1} dilution tube to each of three tubes in a row each containing 10 ml of LTB medium and shake gently so that no air enters inside the Durham tube. Similarly, repeat inoculations as described above for all the dilutions.
- Incubate the inoculated tubes with LTB medium in an incubator at 35 ± 0.5 °C for $24-48 \pm 3$ h.
- After incubation, remove the tubes and check each tube for the presence of growth (turbidity) and gas production inside the Durham tube. If gas production is not readily apparent inside the Durham tube, shake the tubes gently and check for rising gas bubbles. Growth with gas production is considered as positive result for presumptive test.

6.4 *Confirmatory test for FC and E. coli*

- Inoculate the growth from all positive presumptive (LTB) tubes (turbidity with gas production) into EC-MUG media. Using a sterile 3–3.5 mm diameter inoculation loop or sterile wooden applicator stick, take adequate amount of inoculum from each LTB tube with growth and inoculate into separate tubes with EC-MUG medium, shake thoroughly for proper mixing of inoculum.
- Incubate the inoculated EC-MUG media tubes in a water bath/incubator at

44.5 ± 0.2 °C for 24 ± 2 hours. After 24 hours, check each tube for the presence of turbidity and gas production inside the Durham tube. If gas production is not readily apparent, shake the tubes gently and check for rising gas bubbles. Presence of growth (turbidity) with gas production in EC-MUG media is considered as positive result for the confirmation of FC.

- Take all tubes with EC-MUG broth showing growth with or without gas production and examine under long-wavelength UV lamp (preferably 6 W) for fluorescence. The presence of bright blue fluorescence is considered as a positive response for the presence of *E. coli*. A positive control consisting of a known *E. coli* (MUG-positive) culture, a negative culture like thermotolerant *Klebsiella pneumoniae* (MUG-negative) culture, and an uninoculated medium control may be necessary to interpret the results and to avoid confusion of weak autofluorescence of the medium as a positive response.
- Note down the positive (indicated by '+')/negative (indicated by '-') result in each replicate tube of all dilutions of the sample as shown in the Tables 8 and 9.
- Count and note down the total number of positive tubes in each dilution for FC (gas production) and *E. coli* (bright blue fluorescence) separately as shown in the Table 9.

If the result is negative (absence of turbidity with gas production) in LTB medium (Table 8), growth from these tubes is not subcultured and, they are recorded as negative in EC-MUG media also (see Table 9: Recording of results in EC-MUG media).

Only the positive results of confirmatory media (EC-MUG) are taken for estimation/calculation of FC/*E.coli* density according to the procedure shown below in Section 7 (see Table 9: Recording of results in EC-MUG media).

7. Data analysis and calculations

- The estimated density of faecal coliform/*E. coli* bacteria, based on the LTB and

Table 8: Recording of results in LTB media (example)

Sample dilution	10 ⁻¹		10 ⁻²		10 ⁻³		10 ⁻⁴		10 ⁻⁵	
	T	GP	T	GP	T	GP	T	GP	T	GP
EML2023xxxx	T	GP	T	GP	T	GP	T	GP	T	GP
Replicate 1	+	+	+	+	+	+	+	+	∓	∓
Replicate 2	+	+	+	+	+	+	+∓	∓	+	+
Replicate 3	+	+	+	+	+	+	+	+	+	+
Total number of positive tubes in each dilution		3		3		3		2		2

T-turbidity; GP-gas production; *Positive presumptive test result for FC; #Positive result for *E. coli*; ∓gas production is negative in LTB media, hence, growth is not subcultured into EC-MUG media, and result recorded as negative in EC-MUG media for both FC and *E. coli* (see ∓Table 2 below).

Table 9: Recording of results in EC-MUG media (example)

Sample dilution	10 ⁻¹			10 ⁻²			10 ⁻³			10 ⁻⁴			10 ⁻⁵		
EML2023xxxx	T	GP	F	T	GP	F	T	GP	F	T	GP	F	T	GP	F
Replicate 1	+	+	+	+	+	+	+	+	+	+	+	+	∓	∓	∓
Replicate 2	+	+	+	+	+	+	+	+	-	∓	∓	∓	-	-	-
Replicate 3	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-
Total number of positive tubes in each dilution		3*	3#		3*	3#		2*	1#		2*	1#		1*	0#

T: turbidity; GP: gas production; F: bright blue fluorescence; *Positive result for FC; #Positive result for *E. coli*; ∓gas production is negative in LTB media (see ∓Table 1 above), hence, growth is not subculture into EC-MUG media, and result recorded as negative in EC-MUG media for both FC and *E. coli*.

EC-MUG test results, is calculated in terms of most probable number (MPN).

- Faecal coliform/*E. coli* results from biosolid samples are reported as MPN/g total solids (dry weight basis).
- MPN/g total solids (dry weight) is calculated in two steps:

Step 1: Calculation of MPN/mL (wet weight)

Step 2: Conversion to MPN/g total solids (dry weight)

7.1 Step 1: Calculation of MPN/mL (wet weight)

7.1.1 Selection of significant dilutions

- When more than three dilutions of a sample are used in a decimal series of dilutions of a 3-tube MPN determination (see *Table 9: Recording of results in EC-MUG media* and *Table 10: Example calculations including the selection of significant dilutions*); the following guidelines should be followed.
- Results from only three consecutive dilutions are used to determine the MPN.
- If one or more dilutions have all tubes positive, select the highest dilution (smallest sample quantity) with positive results in all tubes and the next two higher dilutions (see examples 'a' and 'b' in *Table 10. Example calculations including the selection of significant dilutions*).
- When none of the dilutions yield all tubes positive, select the three lowest dilutions for which the middle dilution contains the positive result see example 'c' in *Table 10. Example calculations including the selection of significant dilutions*).
- If a positive result occurs in a higher unselected dilution, add the number of positive tubes in this dilution to the results of the highest dilution of the three selected (see example 'd' in *Table 10. Example calculations including the selection of significant dilutions*).

Table 10: Example calculations including the selection of significant dilutions

Example	Sample quantities (g or ml) ¹					Reported positive values	MPN estimate/g or ml
	10	1	0.1	0.01	0.001		
a	3/3 ²	<u>3/3</u>	<u>2/3</u>	<u>0/3</u>	0/3	3-2-0	9.3
b	3/3	3/3	<u>3/3</u>	<u>2/3</u>	<u>0/3</u>	3-2-0	93.
c	0/3	<u>0/3</u>	<u>1/3</u>	<u>0/3</u>	0/3	0-1-0	0.31
d	3/3	<u>3/3</u>	<u>2/3</u>	<u>1/3</u>	1/3	3-2-2	21.
e	3/3	3/3	<u>3/3</u>	<u>3/3</u>	<u>3/3</u>	3-3-3	>1100

¹The analyst should make sure that all sample dilution factors (including the preparation of any sample homogenate) are correctly applied in calculating the actual sample quantities subjected to MPN analysis.

$$\frac{\text{b}^2 \text{ Numerator}}{\text{Denominator}} = \frac{\text{No. positive tubes}}{\text{No. tubes inoculated}}$$

- When all dilutions tested yield all tubes positive, select the three highest dilutions (see example ‘e’ in *Table 10: Example calculations including the selection of significant dilutions*).

7.1.2 Calculation of MPN/mL (wet weight)

- After selecting the significant dilutions/ valid series, the MPN / mL (wet weight) is determined by matching the selected series (also called combination of positives) with the same series (combination of positives) on the MPN reference chart given in Table 11.
- If the selected series (combination of positives) does not match the sample dilution series at the top of the MPN reference chart given in Table 11, the results must be calculated using the following formula:
- MPN / mL (wet weight) = MPN from chart in Table 11 x (ml sample for first column of chart / ml sample in first dilution of the selected series)

7.2 Step 2: Conversion to MPN/g (dry weight):

7.2.1 Determination of total solids

- As the biosolid samples show large variability in their solid content, the MPN is calculated on a dry weight basis.
- For calculation of sample results on a dry weight basis, total solids in the sample have to be determined as follows:
- Take the weight of an empty clean crucible or aluminium evaporating dish (W_1) and tare it.

Table 11: MPN Index and 95% confidence limits for various combinations of positive tubes in a 3 tube dilution series using inoculum quantities of 1, 0.1 and 0.01 g (ml) of original sample

Combination of positives	MPN index per g (ml)	95% Confidence limits	
		Lower	Upper
0-0-0	<0.3	---	0.95
0-0-1	0.30	0.015	0.96
0-1-0	0.30	0.015	1.1
0-1-1	0.61	0.12	1.8
0-2-0	0.62	0.12	1.8
0-3-0	0.94	0.36	3.8
1-0-0	0.36	0.017	1.8
1-0-1	0.72	0.13	1.8
1-0-2	1.1	0.36	3.8
1-1-0	0.74	0.13	2.0
1-1-1	1.1	0.36	3.8
1-2-0	1.1	0.36	4.2
1-2-1	1.5	0.45	4.2
1-3-0	1.6	0.45	4.2
2-0-0	0.92	0.14	3.8
2-0-1	1.4	0.36	4.2
2-0-2	2.0	0.45	4.2
2-1-0	1.5	0.37	4.2
2-1-1	2.0	0.45	4.2
2-1-2	2.7	0.87	9.4
2-2-0	2.1	0.45	4.2
2-2-1	2.8	0.87	9.4
2-2-2	3.5	0.87	9.4
2-3-0	2.9	0.87	9.4
2-3-1	3.6	0.87	9.4
3-0-0	2.3	0.46	9.4
3-0-1	3.8	0.87	11.
3-0-2	6.4	1.7	18.
3-1-0	4.3	0.90	18.
3-1-1	7.5	1.7	20.
3-1-2	12.	3.7	42.
3-1-3	16.	4.0	42.
3-2-0	9.3	1.8	42.
3-2-1	15.	3.7	42.
3-2-2	21.	4.0	43.
3-2-3	29.	9.0	100.
3-3-0	24.	4.2	100.
3-3-1	46.	9.0	200.
3-3-2	110.	18.	410.
3-3-3	>110.	42.	---

- Add 10-30 g of the sample to be tested into the tared crucible or aluminium evaporating dish and note down the weight (W_2). This step should be performed immediately after weighing the sample for microbiological analysis.
- Dry the crucible or aluminium evaporating dish along with the sample overnight in hot-air oven at 103–105 °C.
- After drying, keep the sample in a desiccator and allow it to cool. Take the weight of the crucible with dried sample (W_3).
- Subtract the empty crucible weight (W_1) from the weight of the crucible with dried sample (W_3) to get the weight of dried sample alone (W_4).
- So, $W_4 = W_3 - W_1$
- Calculate the % dry weight of the sample as follows:
- % Dry weight = (weight of dried sample in g [W_4] / weight of sample in g [W_2]) \times 100

Note: The drying oven should be kept in a hood or be vented. Significant laboratory contamination may result from drying a heavily contaminated sample.

MPN / g (dry weight) = [MPN / mL (wet weight) from Step 1 (Section 7.1)] / percent total solids expressed as a decimal

Note 1: FC/*E.coli* can also be tested by ‘5-replicate tube method’, where five replicate tubes per dilution are used instead of three replicate tubes as described in the procedure here. Except the procedure given in ‘Section 7.1’ above, the rest of the procedure remains same for both the methods. For the calculation of ‘MPN/mL (wet weight)’, the procedure and tables given in ‘Annexure 1’ should be followed when using ‘5-replicate tube method’.

Note 2: For ‘5-replicate tube method’, MPN/g (dry weight) = [MPN/mL (wet weight) (calculated as per Annexure 1)]/per cent total solids expressed as a decimal

6.9 STANDARD OPERATING PROCEDURE FOR TESTING OF SALMONELLA IN BIOSOLIDS DERIVED FROM FAECAL SLUDGE BY MODIFIED SEMISOLID RAPPAPORT-VASSILIADIS (MSRV) MEDIUM (US EPA METHOD 1682)

1. Summary, scope and application

Application of treated FS biosolids to land can be helpful as a crop nutrient and soil conditioner but may pose the risk of releasing pathogens into the environment

if proper disinfection and use criteria are not met. Among these organisms are *Salmonella*, which are pathogenic enteric bacteria that can cause salmonellosis in animals and humans, if concentrations able to give rise to infections are present.

The modified semisolid Rappaport-Vassiliadis (MSRV) medium protocol presented in EPA method 1682 provides enumeration of *Salmonella* in biosolids based on the most probable number (MPN) technique. The determination of *Salmonella* involves inoculating the enrichment medium, tryptic soy broth (TSB), with a measured amount of sample and incubating for 24 hours. After incubation, TSB is spotted onto the selective MSRV medium. The MSRV medium uses novobiocin and malachite green to inhibit non-*Salmonella* species, while allowing most *Salmonella* species to grow. Presumptively identified colonies are isolated on xylose-lysine desoxycholate agar (XLD), and confirmed using lysine-iron agar (LIA), triple sugar iron agar (TSI), and urea broth, followed by positive serological typing using polyvalent O antisera. A total solid (% dry weight) determination is performed on a representative biosolid sample and is used to calculate MPN / g dry weight. *Salmonella* density is reported as MPN/4 g dry weight.

2. Equipment and supplies

- Latex gloves;
- Erlenmeyer flasks, 1 L and 2 L;
- Measuring cylinders, 1 L;
- Weighing balance; butter paper;
- Microwave oven/hot plate; screw cap bottle, borosilicate glass, 500 ml;
- Culture media dispensing pump;
- Test tubes, rimless, borosilicate glass (25 x 150 mm, 18 x 150 mm, 15 x 150 mm);
- Loose-fitting autoclavable plastic caps (25 mm, 18 mm, 15 mm diameter);
- Test tube stands, autoclavable (16 mm, 20 mm, 25 mm diameter);
- Test tube basket, autoclavable, 180 x 170 x 160 mm;
- Autoclave;
- Plastic sterile petri dishes, 15 x 100 mm;
- Petri dish, borosilicate glass, 15 x 100 mm;
- Aluminium foil or crucible;
- Laboratory blender;
- Wide mouth bottle, polypropylene, autoclavable, 500 ml;
- Biosafety cabinet/laminar airflow;
- Bunsen burner or alcohol burner;
- Vortex mixer;
- Micropipettes and tips;
- or 3.5 mm diameter inoculation loop;

-
- Incubator/water bath;
 - Glass slides for agglutination test;
 - Glass cover slips;
 - Magnifying glass or dissection scope.

3. Reagents

- Distilled water;
- Tryptic soy broth (TSB) (himedia);
- Modified semisolid Rappaport- Vassiliadis (MSRV) medium (himedia);
- Novobiocin (himedia);
- Xylose-lysine desoxycholate agar (XLD) (himedia);
- Triple sugar iron agar (TSI) (himedia);
- Lysine iron agar (LIA) (himedia);
- Urea broth (himedia);
- *Salmonella* O antiserum polyvalent groups A-I and Vi (BD Difco).

4. Reagent preparation

4.1 *Water for serial dilution*

- Pour distilled water into the 500 mL screwcap bottle fitted with a culture media dispensing pump.
- Dispense 9 ml of distilled water into 18 x 150 mm tubes using the culture media dispensing pump.
- Close the tubes with 18 mm autoclavable loose fitting plastic caps, place them in a test tube basket or test tube stand and sterilize in an autoclave at 121°C temperature (15 PSI pressure) for 15 minutes.

4.2 *Culture media*

4.2.1 Tryptic soy broth (TSB)

- For single strength (1X) TSB, add 30 g of dehydrated TSB media powder to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve.
- Dispense 10 mL volumes into 15 × 150 mm culture tubes. The 1X TSB will be used for inoculation volumes of 1 mL. Autoclave for 15 minutes at 121°C (15 PSI).
- For triple strength (3X) TSB, use 333 mL of reagent-grade water instead of 1 L. Dispense 10 mL and 5 mL volumes into 25 × 150 mm culture tubes. The 3X TSB tubes containing 10 mL of media has to be inoculated with 20 mL of homogenized sample. The 3X TSB tubes containing 5 mL of media has to be inoculated with 10 mL of homogenized sample. Autoclave for 15 minutes at 121°C (15 PSI). Cool the media to room temperature before use.

- Note: 3X TSB is necessary for 20- and 10-mL inoculations to ensure that the inoculation volume does not excessively dilute the media.

4.2.2 Modified semisolid Rappaport-Vassiliadis (MSRV) medium

- Add 15.83 g of dehydrated MSRV media powder to 500 ml of reagent-grade water, mix thoroughly, and heat to boiling to dissolve completely (do not autoclave).
- Cool to 50°C, and add 1.0 mL of a 2% stock solution of novobiocin per litre of medium. Mix well by swirling the medium. Immediately pour approximately 25 mL into 15 × 100 mm petri plates. Do not invert plates to store.
- Note: If using a commercially prepared novobiocin antimicrobial supplement, add sufficient volume to achieve a concentration of 0.002 per cent per litre.

4.2.3 Xylose-lysine desoxycholate agar (XLD)

- Add 56.68 g of dehydrated XLD media to 1 L of reagent-grade water, mix thoroughly, and heat to boiling to dissolve completely, avoid overheating (do not autoclave).
- Cool to 45–50°C and immediately pour approximately 12 mL into 15 × 100 mm sterile petri plates. Cool the media to room temperature prior to inoculation.

4.2.4 Triple sugar iron agar (TSI)

- Add 64.52 g of dehydrated TSI media to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely.
- Dispense 5–7 mL aliquots into 16 × 100 mm screw cap test tubes, cap and autoclave at 121°C (15 PSI) for 15 minutes.
- Allow medium to solidify in a slant rack or rack that is tilted in such a manner that the surface area is equally divided between the slant and butt. Let the media cool to room temperature prior to inoculation.

4.2.5 Lysine iron agar (LIA)

- Add 34.56 g of dehydrated LIA medium (HiMedia) in 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely.
- Dispense 5–7 mL aliquots into 16 × 100 mm screw cap test tubes, cap and autoclave at 121°C (15 PSI) for 12 minutes.
- Allow medium to solidify in a slant rack or rack that is tilted in such a manner that the surface area is equally divided between the slant and butt. Let the media cool to room temperature prior to inoculation.

4.2.6 Urea broth

- Add 18.71 g dehydrated urea broth base medium (HiMedia) in 950 ml of reagent-grade water, mix thoroughly to dissolve (do not boil or autoclave).
- Add 50 mL of sterile 40% urea solution (HiMedia) to the 950 mL of urea broth base and mix.
- Filter sterilize by passing solution through a sterile, 0.22 µm filter into a sterile flask (Urea broth can be prepared in volumes of 100–200 mL for easy sterilization by membrane filtration.)
- Aseptically dispense 3 mL into sterile 16 × 100 mm screw cap test tubes using a sterile pipet or sterile dispensing syringe. Let the media cool to room temperature prior to inoculation.

4.3 *Salmonella* 'O' antiserum polyvalent groups A-I and Vi

5. Control organisms

5.1 Positive controls

Obtain a stock culture of *Salmonella typhimurium* ATCC # 14028 as a positive control for MSR/V, XLD, TSI, LIA, and polyvalent O antiserum. Obtain a stock culture of *Proteus vulgaris* ATCC # 13315 as a positive control for urease.

5.2 Negative controls

Obtain a stock culture of *Escherichia coli* ATCC # 25922 as a negative control for MSR/V, XLD, TSI, LIA, and polyvalent O antiserum. Obtain a stock culture of *Salmonella typhimurium* ATCC # 14028 as a negative control for urease.

6. Procedure

6.1 Sample preparation/sample homogenization

- Mix the sample thoroughly and weigh 30.0 ± 0.1 g in a sterile petri dish/aluminium foil. If the sample has smaller solid materials like wooden chips etc., crush and grind them using mortar and pestle before weighing to obtain a homogeneous mixture. Larger and harder substances that are difficult to ground can be discarded.
- Add the sample into sterile laboratory blender jar.
- Add the 270 mL sterile distilled water into the blender jar, close the jar with lid and blend at high speed for one to two minutes to obtain 'homogenized' sample. Each mL of the homogenized sample contains 10^{-1} (0.1) g of the original sample. Adjust the pH to 7.0-7.5 using 1.0 N HCl or 1.0 N NaOH, if required.

6.2 *Enrichment phase*

- For each sample, arrange test tubes in three rows/series of five tubes each. As five replications are used per dilution/series, this is also called as '5-tube method'. Three series of five tubes will be used for the analysis with 2.0, 1.0, and 0.1 g of the original sample (20.0, 10.0, and 1.0 mL of the homogenized sample). The first two series of tubes must contain 3X TSB.
- Using a sterile pipette, inoculate each of the first series of tubes (containing 10 mL of 3X TSB) with 20.0 mL of the 'homogenized' sample per tube. This is 2.0 g of the original sample.
- Using a sterile pipette, inoculate each of the second series of tubes (containing 5 mL of 3X TSB) with 10.0 mL of the 'homogenized' sample per tube. This is 1.0 g of the original sample.
- Using a sterile pipette, inoculate each of the third series of tubes (containing 10 mL of 1X TSB) with 1.0 mL of the 'homogenized' sample per tube. This is 0.1 g of the original sample.
- If the sample is suspected to contain high concentrations of *Salmonella*, then inoculate 1 X TSB media with three additional sample dilutions (series) or more (0.1 mL, 0.01 mL, 0.001 mL and so on of the homogenized sample per replicate tube which contains 0.01 g, 0.001 g, 0.0001 g and so on respectively of the original sample) of five tubes each.
- Incubate the TSB tubes and controls for 24 ± 2 hours at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$.
- Record all turbid tubes as positive. Because of the non-inhibitory nature of the enrichment medium, all tubes will be positive in most instances. If none of the tubes appear to be positive, then this may indicate the presence of a toxic substance or that the tubes were not inoculated.

6.3 *Selection phase*

- Apply six discrete, 30- μL drops from each TSB tube onto a corresponding MSR/V plate that has been labeled with sample ID, date, and original inoculation volume (e.g. 20.0, 10.0, 1.0 mL, or so on).
- Space the drops evenly over the entire plate. In addition, inoculate an MSR/V plate with positive and negative controls. Do not invert the plates. Allow the drops to absorb into the agar for approximately one hour at room temperature and incubate plates at $42^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 16 to 18 hours in a humidity-controlled hot air incubator. If a humidity-controlled hot air incubator is not available, an open pan of water placed in the bottom of the incubator will suffice.
- Examine plates for the appearance of motility surrounding the inoculations, as evidenced by a 'whitish halo' of growth approximately 2 cm from the center of the spot.
- Using a sterile inoculating loop, stab into a halo from the outer edge of a target

colony on the MSR/V plate and streak onto an XLD plate. Since *Salmonella* are predominately located within the MSR/V media, the loop should penetrate the MSR/V at least half-way. Repeat this step using another target colony from the MSR/V plate. In addition, inoculate XLD positive and negative controls.

- Incubate XLD plates for 18 to 24 hours at $36\text{ }^{\circ}\text{C} \pm 1.5\text{ }^{\circ}\text{C}$. Black and pink to red colonies with black centres are considered *Salmonella*.
- Note: H_2S negative *Salmonella* will be missed if translucent pink to red colonies are not submitted to biochemical and serological confirmation.

6.4 Biochemical confirmation phase

- Label all tubes with inoculation date, sample identification, and original inoculation volume (e.g. 20.0, 10.0, 1.0 mL or so on). Pick isolated colonies exhibiting *Salmonella* morphology (pink to red colonies with black centers) and inoculate triple sugar iron agar (TSI) slants, lysine iron agar (LIA) slants, and urea broth. Inoculate slants by stabbing the butt and streaking the slant. Use the same XLD colony to inoculate all three media. This will require going back to the same XLD colony multiple times to ensure sufficient inoculum for each medium. In addition, inoculate each medium with the appropriate positive and negative controls.
- Incubate for 24 ± 2 hours at $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$.
- If only atypical colony morphology is observed on XLD plates, pick from an atypical colony and inoculate TSI slants, LIA slants, and urea broth and incubate as described above.

6.4.1 TSI:

- A positive TSI reaction is an acid butt (yellow in color) and an alkaline slant (red in color) with or without H_2S gas production.
- When H_2S gas production is present, the butt of TSI may be black, which would be considered a positive reaction for *Salmonella*.
- H_2S presence is more likely but acid butt is also possible (but rare).

6.4.2 LIA:

- A positive LIA reaction is an alkaline butt (purple in colour) and an alkaline slant (purple in colour) with or without H_2S gas production.
- When H_2S gas production is present, the butt of LIA may be black, which would be considered a positive reaction for *Salmonella*.

6.4.3 Urea broth:

- Urea is an orange medium and will change to pink or deep purplish-red if positive.

- A negative urease test is one that exhibits no colour change after inoculation. *Salmonella* is negative for urease.

6.5 Serological confirmation

- To confirm cultures via polyvalent O antiserum, emulsify growth on the slant portion of TSI (regardless of whether TSI is positive or negative) using sterile physiological saline.
- Place two discrete drops of emulsified growth onto the slide.
- To the first drop of emulsified growth, add one drop of polyvalent O antiserum.
- To the second drop of emulsified growth, add one drop of sterile saline (as a visual comparison).
- Observe under magnification for an agglutination reaction which indicates a positive result.
- Appropriate positive and negative controls from TSI must be analysed for each batch of samples.

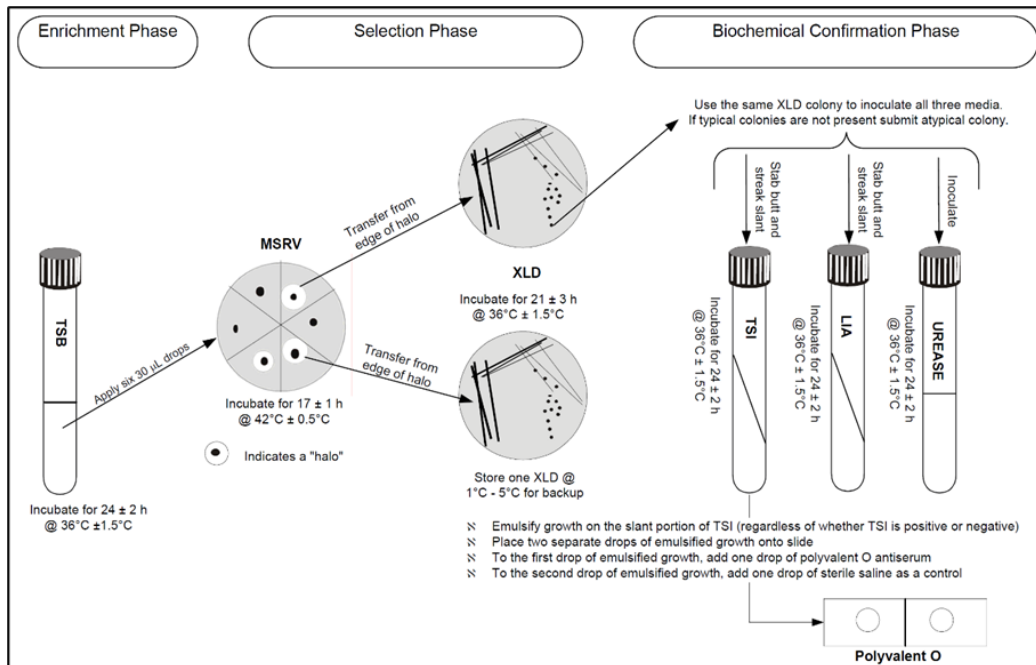
In order for the original TSB tube to be considered positive for *Salmonella*, the associated inoculations must be MSRV positive, XLD positive, either TSI or LIA positive, urease negative, and Polyvalent-O positive (see *Table 12: Positive and negative control results in various media and antisera*). Correlate all positive plates and tubes to original TSB tube and record results. An overview of the US EPA Method 1682 (see *Fig. 2: Method 1682 MSRV procedure*). Determine the MPN from these results by the procedure given in Section 7.

Table 12: Positive and negative control results in various media and antisera

Medium	<i>Salmonella</i> result	Positive method 1682 reaction	Negative method 1682 reaction
Tryptic soy broth (TSB)	Positive	Turbidity	No turbidity
Modified semisolid Rappaport-Vassiliadis (MSRV) medium	Positive	Migrated cells visible as a gray- white turbid zone (halo) extending out from inoculations	Medium remains blue-green around inoculations with no gray-white turbid zone (halo) (<i>E. coli</i> has marked inhibition)
Xylose lysine desoxycholate agar (XLD)	Positive	Pink to red colonies with black centers	Other colours with or without black centers (e.g. <i>E. coli</i> is yellow without black centre)
Triple sugar iron agar (TSI)	Positive	Good growth with alkaline slant (red) with acid butt (yellow) with or without H ₂ S production (which may result in a black butt)	Other colour combinations (e.g., <i>E. coli</i> is yellow slant and butt)
Lysine iron agar (LIA)	Positive	Alkaline slant (purple) with alkaline butt (purple) with or without H ₂ S production (which may result in a black butt)	Other color combinations (e.g. <i>E. coli</i> is red to red/purple slant and butt without H ₂ S production)
Urea broth	Negative	Pink	No colour change (<i>Salmonella</i> is urease negative)
Polyvalent O antisera	Positive	Agglutination	No agglutination

7. Data analysis and calculations

Figure 2: Method 1682 MSRV procedure (repeat the following for each TSB tube)



- The estimated density of *Salmonella*, based on the above results, is calculated in terms of most probable number (MPN) as given in the US EPA Method 1682.
- Due to the variability in the solid content of the biosolid sample derived from fecal sludge, *Salmonella* results from these samples are expressed as MPN/g total solids (dry weight basis).
- MPN/g total solids (dry weight) is calculated in two steps:

Step 1: Calculation of MPN/mL (wet weight)

Step 2: Conversion to MPN/g total solids (dry weight)

7.1 Step 1: Calculation of MPN/mL (wet weight)

- Obtain the MPN index value from Table 13 using the number of positive tubes in the three significant dilutions series.
- If only 20.0, 10.0, and 1.0 mL of homogenized sample were inoculated (in 5 replicate tubes each), the 'MPN index from Table 13' is the 'MPN/ mL (wet weight) of homogenized sample'. This is because, Table 13 assumes that

20.0, 10.0, and 1.0 mL of homogenized sample were inoculated into TSB in 5 replicate tubes each.

- When additional dilution volumes of 0.1-, 0.01-, and 0.001-mL, or more of homogenized sample are inoculated (in 5 replicate tubes each) for quantifying FS biosolid samples expected to contain higher *Salmonella* concentrations, the procedure given in Annexure 1 should be followed to obtain the MPN/mL (wet weight).
- Since FS biosolid samples were diluted in the homogenization step (Section 6.1), the dilution must be taken into account when calculating MPN/mL (wet weight). As a result, the 'MPN index value from Table 13/ Table A1.2 in Annexure 1' is divided by 0.1 to account for diluting the sample during the homogenization step.

Table 13: MPN index and 95% confidence limits for various combinations of positive results when five tubes are used per 20.0, 10.0, and 1.0 mL homogenized sample inoculation volumes*

Combination of positives	MPN Index	95% confidence limits		Combination of positives	MPN Index	95% confidence limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.006473	----	0.0223	1-3-0	0.0312	0.0055	0.0678
0-0-1	0.0065	0.0012	0.0223	1-3-1	0.0393	0.0092	0.0821
0-0-2	0.0130	0.0012	0.0352	1-3-2	0.0475	0.0132	0.0967
0-0-3	0.0195	0.0012	0.0472	1-3-3	0.0559	0.0173	0.1119
0-0-4	0.0262	0.0033	0.0589	1-3-4	0.0644	0.0216	0.1277
0-0-5	0.0328	0.0062	0.0706	1-3-5	0.0730	0.0260	0.1444
0-1-0	0.0067	0.0012	0.0228	1-4-0	0.0409	0.0099	0.0849
0-1-1	0.0134	0.0012	0.0360	1-4-1	0.0495	0.0141	0.1002
0-1-2	0.0202	0.0012	0.0483	1-4-2	0.0583	0.0185	0.1163
0-1-3	0.0270	0.0037	0.0604	1-4-3	0.0672	0.0231	0.1331
0-1-4	0.0339	0.0067	0.0725	1-4-4	0.0763	0.0277	0.1509
0-1-5	0.0408	0.0099	0.0847	1-4-5	0.0855	0.0324	0.1700
0-2-0	0.0138	0.0012	0.0367	1-5-0	0.0517	0.0152	0.1042
0-2-1	0.0208	0.0012	0.0495	1-5-1	0.0609	0.0199	0.1212
0-2-2	0.0279	0.0040	0.0619	1-5-2	0.0703	0.0247	0.1391
0-2-3	0.0350	0.0072	0.0745	1-5-3	0.0799	0.0296	0.1583
0-2-4	0.0422	0.0106	0.0871	1-5-4	0.0897	0.0346	0.1790
0-2-5	0.0494	0.0141	0.1001	1-5-5	0.0998	0.0397	0.2015
0-3-0	0.0215	0.0012	0.0507	2-0-0	0.0155	0.0012	0.0404
0-3-1	0.0288	0.0044	0.0636	2-0-1	0.0226	0.0018	0.0526
0-3-2	0.0362	0.0077	0.0766	2-0-2	0.0303	0.0051	0.0662
0-3-3	0.0437	0.0113	0.0898	2-0-3	0.0382	0.0087	0.0801
0-3-4	0.0512	0.0051	0.1243	2-0-4	0.0462	0.0125	0.0943

Combination of positives	MPN Index	95% confidence limits		Combination of positives	MPN Index	95% confidence limits	
		Lower	Upper			Lower	Upper
0-3-5	0.0588	0.0095	0.1428	2-0-5	0.0543	0.0165	0.1090
0-4-0	0.0299	0.0049	0.0654	2-1-0	0.0234	0.0022	0.0540
0-4-1	0.0375	0.0084	0.0789	2-1-1	0.0315	0.0056	0.0683
0-4-2	0.0453	0.0121	0.0927	2-1-2	0.0397	0.0094	0.0827
0-4-3	0.0531	0.0160	0.1069	2-1-3	0.0480	0.0134	0.0976
0-4-4	0.0611	0.0200	0.1216	2-1-4	0.0565	0.0177	0.1131
0-4-5	0.0691	0.0241	0.1369	2-1-5	0.0652	0.0221	0.1293
0-5-0	0.0390	0.0090	0.0814	2-2-0	0.0327	0.0062	0.0705
0-5-1	0.0470	0.0129	0.0958	2-2-1	0.0413	0.0101	0.0856
0-5-2	0.0553	0.0170	0.1107	2-2-2	0.0501	0.0144	0.1013
0-5-3	0.0636	0.0212	0.1262	2-2-3	0.0590	0.0189	0.1176
0-5-4	0.0720	0.0255	0.1425	2-2-4	0.0681	0.0236	0.1349
0-5-5	0.0806	0.0299	0.1596	2-2-5	0.0774	0.0283	0.1533
1-0-0	0.0072	0.0012	0.0241	2-3-0	0.0431	0.0110	0.0887
1-0-1	0.0139	0.0012	0.0369	2-3-1	0.0523	0.0155	0.1053
1-0-2	0.0209	0.0012	0.0497	2-3-2	0.0617	0.0203	0.1227
1-0-3	0.0281	0.0041	0.0623	2-3-3	0.0714	0.0252	0.1412
1-0-4	0.0353	0.0073	0.0749	2-3-4	0.0813	0.0303	0.1611
1-0-5	0.0425	0.0107	0.0878	2-3-5	0.0914	0.0354	0.1826
1-1-0	0.0144	0.0012	0.0377	2-4-0	0.0547	0.0168	0.1098
1-1-1	0.0217	0.0013	0.0509	2-4-1	0.0647	0.0218	0.1284
1-1-2	0.0290	0.0045	0.0640	2-4-2	0.0750	0.0271	0.1484
1-1-3	0.0365	0.0079	0.0771	2-4-3	0.0855	0.0325	0.1700
1-1-4	0.0441	0.0115	0.0905	2-4-4	0.0964	0.0380	0.1937
1-1-5	0.0517	0.0153	0.1043	2-4-5	0.1076	0.0436	0.2201
1-2-0	0.0224	0.0017	0.0523	2-5-0	0.0681	0.0235	0.1349
1-2-1	0.0301	0.0050	0.0658	2-5-1	0.0791	0.0292	0.1566
1-2-2	0.0379	0.0085	0.0795	2-5-2	0.0904	0.0349	0.1805
1-2-3	0.0457	0.0123	0.0935	2-5-3	0.1021	0.0409	0.2070
1-2-4	0.0537	0.0162	0.1079	2-5-4	0.1143	0.0469	0.2372
1-2-5	0.0618	0.0203	0.1229	2-5-5	0.1268	0.0531	0.2725
3-0-0	0.0255	0.0028	0.0585	4-3-0	0.0797	0.0295	0.1579
3-0-1	0.0330	0.0063	0.0710	4-3-1	0.0937	0.0366	0.1877
3-0-2	0.0417	0.0103	0.0863	4-3-2	0.1086	0.0441	0.2228
3-0-3	0.0506	0.0147	0.1023	4-3-3	0.1245	0.0520	0.2656
3-0-4	0.0598	0.0193	0.1191	4-3-4	0.1414	0.0602	0.3218
3-0-5	0.0691	0.0241	0.1368	4-3-5	0.1595	0.0686	0.4067
3-1-0	0.0344	0.0069	0.0734	4-4-0	0.1012	0.0404	0.2049
3-1-1	0.0435	0.0112	0.0896	4-4-1	0.1181	0.0489	0.2476
3-1-2	0.0529	0.0159	0.1065	4-4-2	0.1364	0.0578	0.3038

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Combination of positives	MPN Index	95% confidence limits		Combination of positives	MPN Index	95% confidence limits	
		Lower	Upper			Lower	Upper
3-1-3	0.0626	0.0207	0.1244	4-4-3	0.1563	0.0672	0.3890
3-1-4	0.0725	0.0258	0.1434	4-4-4	0.1780	0.0770	0.5273
3-1-5	0.0827	0.0310	0.1640	4-4-5	0.2015	0.0873	0.6411
3-2-0	0.0456	0.0122	0.0932	4-5-0	0.1304	0.0549	0.2836
3-2-1	0.0555	0.0171	0.1112	4-5-1	0.1524	0.0653	0.3687
3-2-2	0.0657	0.0223	0.1303	4-5-2	0.1769	0.0766	0.5210
3-2-3	0.0763	0.0277	0.1510	4-5-3	0.2046	0.0886	0.6528
3-2-4	0.0872	0.0333	0.1735	4-5-4	0.2357	0.1015	0.7516
3-2-5	0.0984	0.0390	0.1984	4-5-5	0.2708	0.1150	0.8426
3-3-0	0.0583	0.0186	0.1164	5-0-0	0.0549	0.0162	0.1116
3-3-1	0.0693	0.0241	0.1371	5-0-1	0.0637	0.0213	0.1265
3-3-2	0.0806	0.0299	0.1597	5-0-2	0.0763	0.0277	0.1510
3-3-3	0.0924	0.0359	0.1847	5-0-3	0.0896	0.0345	0.1787
3-3-4	0.1046	0.0421	0.2128	5-0-4	0.1037	0.0417	0.2107
3-3-5	0.1173	0.0484	0.2452	5-0-5	0.0953	0.0165	0.2234
3-4-0	0.0733	0.0262	0.1450	5-1-0	0.0678	0.0234	0.1344
3-4-1	0.0856	0.0325	0.1700	5-1-1	0.0816	0.0304	0.1618
3-4-2	0.0984	0.0390	0.1982	5-1-2	0.0963	0.0379	0.1936
3-4-3	0.1118	0.0457	0.2307	5-1-3	0.1121	0.0459	0.2316
3-4-4	0.1258	0.0526	0.2695	5-1-4	0.1291	0.0542	0.2796
3-4-5	0.1405	0.0597	0.3184	5-1-5	0.1293	0.0304	0.3090
3-5-0	0.0913	0.0354	0.1825	5-2-0	0.0879	0.0337	0.1751
3-5-1	0.1055	0.0426	0.2150	5-2-1	0.1046	0.0421	0.2128
3-5-2	0.1204	0.0500	0.2538	5-2-2	0.1227	0.0511	0.2605
3-5-3	0.1362	0.0577	0.3029	5-2-3	0.1427	0.0608	0.3267
3-5-4	0.1529	0.0656	0.3715	5-2-4	0.1646	0.0710	0.4385
3-5-5	0.1707	0.0738	0.4795	5-2-5	0.1767	0.0503	0.5230
4-0-0	0.0381	0.0082	0.0809	5-3-0	0.1151	0.0474	0.2394
4-0-1	0.0461	0.0125	0.0942	5-3-1	0.1368	0.0580	0.3050
4-0-2	0.0563	0.0175	0.1126	5-3-2	0.1614	0.0695	0.4183
4-0-3	0.0668	0.0229	0.1323	5-3-3	0.1895	0.0821	0.5899
4-0-4	0.0777	0.0284	0.1537	5-3-4	0.2216	0.0957	0.7101
4-0-5	0.0890	0.0342	0.1773	5-3-5	0.2527	0.0814	0.7971
4-1-0	0.0484	0.0136	0.0983	5-4-0	0.1571	0.0676	0.3935
4-1-1	0.0592	0.0190	0.1181	5-4-1	0.1907	0.0826	0.5954
4-1-2	0.0705	0.0248	0.1395	5-4-2	0.2319	0.0999	0.7409
4-1-3	0.0822	0.0308	0.1631	5-4-3	0.2834	0.1196	0.8726
4-1-4	0.0945	0.0370	0.1894	5-4-4	0.3475	0.1417	1.0160
4-1-5	0.1072	0.0434	0.2193	5-4-5	0.4256	0.1437	1.1800
4-2-0	0.0626	0.0207	0.1244	5-5-0	0.2398	0.0762	0.7629

Combination of positives	MPN Index	95% confidence limits		Combination of positives	MPN Index	95% confidence limits	
		Lower	Upper			Lower	Upper
4-2-1	0.0748	0.0269	0.1479	5-5-1	0.3477	0.1172	1.0160
4-2-2	0.0875	0.0335	0.1742	5-5-2	0.5422	0.1791	1.4190
4-2-3	0.1009	0.0403	0.2041	5-5-3	0.9178	0.2672	2.2010
4-2-4	0.1150	0.0473	0.2392	5-5-4	1.6090	0.3837	4.1030
4-2-5	0.1299	0.0546	0.2820	5-5-5	>1.609000	0.3837	----

*Table 2 was developed using the MPN calculator developed by Albert Klee (1993).

7.2 Step 2: Conversion to MPN/g (dry weight):

7.2.1 Determination of total solids

Determine the total solids as per the method described in protocol 6.8, Section 7.2.1.

For the conversion to MPN / g total solids (dry weight), we assume that, MPN / mL (wet weight) = MPN / g (wet weight).

So, MPN/g (dry weight) = [MPN / mL (wet weight) from Step 1 (Section 7.1)] / per cent total solids expressed as a decimal

Note: As *Salmonella* in biosolids is reported in MPN/4g (dry solids) (as per the units of available standards for *Salmonella* in biosolids), the following calculation is given for conversion to MPN/4g (dry solids).

MPN / 4 g (dry weight) =

[MPN / mL (wet weight) from Step 1 (Section 7.1)] x 4 Per cent total solids expressed as a decimal

Note: Testing of *Salmonella* in FS biosolid by 3-replicate tube method:

- *Salmonella* can also be tested by the 3-replicate tube method, where three replicate tubes per dilution are used instead of five replicate tubes as described in the above procedure. Except the procedure (and MPN reference charts) given/referred to in Section 7.1 above, the rest of the procedure remains same for both the methods.
- For the calculation of MPN/mL (wet weight), the procedure and tables given in Section 7.1 of procedure for 'Testing of faecal coliform and *Escherichia coli* in faecal sludge derived biosolid by most probable method (MPN) method' should be followed when using the 3-replicate tube method. However, 'MPN reference chart' is not available when '20.0 mL' sample volume is used in a 3-replicate

tube method. In this case, the online MPN calculator available at <https://mostprobablenumbercalculator.epa.gov/mpnForm> provided by US EPA can be used. Since FS biosolid samples were diluted in the homogenization step (Section 6.1 above), the dilution must be taken into account when calculating MPN/mL (wet weight). As a result, the 'MPN index' (MPN/mL) calculated using online calculator should be divided by 0.1 to account for diluting the sample during the homogenization step.

6.10 STANDARD OPERATING PROCEDURE FOR THE TESTING OF SALMONELLA IN BIOSOLIDS DERIVED FROM FAECAL SLUDGE BY PLATE-COUNT METHOD USING HICROME SALMONELLA AGAR

1. Summary

Conventional method employs the H₂S production property for *Salmonella* detection which is also exhibited by other non-*Salmonella* species such as *Citrobacter*, *Proteus*, etc. HiCrome™ *Salmonella* Agar differentiates *Salmonella* based on propylene glycol utilization and presence of a chromogenic indicator. *Escherichia coli* and *Salmonella* are easily distinguishable due to their colony characteristics. *Salmonella* forms purple-coloured colonies with a purple halo. *E.coli* and other β-glucuronidase positive organism exhibits a characteristic blue colour. Bile salts mixture containing sodium deoxycholate inhibits gram-positive organisms.

2. Principle

HiCrome *Salmonella* agar containing bile salts, chromogenic mixture and propylene glycol in the supplement makes it possible to differentiate *Salmonella* species. Bile salts mixture containing sodium deoxycholate inhibits Gram-positive organisms. *Salmonella* form acid due to the fermentation of propylene glycol. The presence of a pH indicator (chromogenic mixture) gives the colonies purple colour with a halo. *E.coli* possess the enzyme -glucuronidase that splits the bond between the chromophore and the glucuronide (chromogenic mixture). The released chromophore gives blue coloration to the colonies. Other *Enterobacteriaceae* and gram-negative bacteria, such as *Proteus*, *Pseudomonas*, *Shigella*, *S. typhi* and *S. paratyphi A* grow as colourless colonies.

3. Apparatus

- Latex gloves;
- Conical flask;
- Measuring cylinder;
- Weighing balance;

-
- Butter paper;
 - Microwave oven;
 - Screw cap bottle, borosilicate glass, 500 ml;
 - Culture media dispensing pump;
 - Test tubes, rimless, borosilicate glass (18 × 150 mm);
 - Loose- fitting autoclavable plastic caps (18 mm diameter);
 - Test tube stands, autoclavable (20 mm diameter);
 - Test tube basket, autoclavable, 180 x 170 x 160 mm;
 - Autoclave;
 - Sterile petri dishes, borosilicate glass, 15 x 100 mm;
 - Aluminium foil or crucible;
 - Laboratory blender;
 - Wide mouth bottle, polypropylene, autoclavable, 500 ml;
 - Biosafety cabinet/laminar airflow;
 - Vortex mixer;
 - Micropipettes and tips;
 - Spreader;
 - 70% ethanol;
 - Bunsen burner or alcohol burner;
 - Incubator.

4. Reagents

- Distilled water;
- HiCrome *Salmonella* Agar (HSA) (HiMedia)

5. Reagent preparation

5.1 Water for serial dilution

- Pour distilled water into the 500 mL screw cap bottle fitted with a culture media dispensing pump. Dispense 9 ml of distilled water into 18 x 150 mm tubes using the culture media dispensing pump.
- Close the tubes with 18 mm autoclavable loose fitting plastic caps, place them in a test tube basket or test tube stand and sterilize in an autoclave at 121°C temperature (15 psi pressure) for 15 minutes.

5.2 HiCrome Salmonella Agar (HSA) plates

- Add 27.9 g of dehydrated HSA media powder to 1000 ml of reagent-grade water, mix thoroughly, and gently heat to boiling to dissolve the media completely (do not autoclave).

- Cool to 45–50°C and immediately pour approximately 15 mL into 15 × 100 mm sterile petri plates. Allow the media to solidify. Let the media reach to room temperature prior to inoculation.
- Note: Pouring HSA media for solidification in plates prior to inoculation is required only for spread plate method. For pour plate technique, melted HSA media should be maintained in liquid state by keeping in an incubator at > 50°C. This media, after cooling to appropriate temperature, is poured into petri plate with prior inoculum and then allowed to solidify.

6. Procedure

6.1 Sample preparation/ sample homogenization

- Mix the sample thoroughly and weigh 30.0 ± 0.1 g in a sterile petri dish/ aluminium foil. If the sample has smaller solid materials like wooden chips etc., crush and grind them using mortar and pestle before weighing to obtain a homogeneous mixture. Larger and harder substances that are difficult to ground can be discarded.
- Add the sample into sterile laboratory blender jar.
- Add the 270 mL sterile distilled water into the blender jar, close the jar with lid and blend at high speed for 1–2 minutes. This is the ‘homogenized’ sample. Each mL of the ‘homogenized’ sample contains 10^{-1} (0.1) g of the original sample. Adjust the pH to 7.0–7.5 using 1.0 N HCl or 1.0 N NaOH, if required.

6.2 Sample dilution

- The determination of *Salmonella* by plate-count method requires prior dilution of the samples. The extent of dilution depends on the type of sample being analysed. In the treated biosolid samples of faecal origin, the *Salmonella* count will vary depending on the extent of treatment (up to 10^{-5} or more).
- To take adequate quantity of original sample for analysis, at the initial step of sample dilution, it is recommended to always add 10 mL of ‘homogenized sample’ to 90 mL of sterile dilution water and shake vigorously at least 25 times. This will dilute the original sample 10^{-2} times i.e., each 1 mL aliquot contains 10^{-2} [0.01] g of original sample. Label this dilution as ‘ 10^{-2} ’. Note: If 10 ml of sample is not taken for analysis, then proceed as follows: Take 1 mL aliquot from this bottle and add to tube containing 9ffl 0.2 mL sterile dilution water and mix by vortexing. This tube contains ‘ 10^{-2} g’ of original sample. Label this dilution as ‘ 10^{-2} ’.
- Take 1 ml aliquot from the tube with ‘ 10^{-2} g’ of original sample and add to sterile tube containing 9 ± 0.2 mL sterile dilution water and mix by vortexing. This tube contains ‘ 10^{-3} g’ of original sample. Label this dilution as ‘ 10^{-3} ’.

- Take 1 ml aliquot from the tube with '10⁻³ g' of original sample and add to sterile tube containing 9 ± 0.2 mL sterile dilution water and mix by vortexing. This tube contains '10⁻⁴ g' of original sample. Label this dilution as '10⁻⁴'. Depending on requirement, serially dilute the sample as done in this step and label the dilutions as '10⁻⁵', '10⁻⁶', and so on.
- Do not suspend sample in dilution water for more than 30 min at room temperature to avoid death or multiplication of microorganisms.
- Use sterile pipette and tips at each step of dilution and inoculation to avoid cross contamination.

6.3 Inoculation

- Two methods are available for testing *Salmonella* in FS biosolids using plate-count technique: Pour plate and spread plate methods.
- Depending on the suspected concentration of *Salmonella* in the FS biosolid sample, the dilutions for inoculation should be decided. It is recommended to inoculate three different (consecutive) dilutions of a sample for getting optimum colony count.

6.3.1 Pour plate method

- Label media name, sample ID, sample dilution (eg. 10⁻¹, 10⁻², and 10⁻³) and type of plate-count technique used (pour plate) on the base of each empty sterile petri plate without media.
- Add 1.0 mL of aliquot from each of three selected dilutions into separate empty petri dishes without media, e.g. 1 mL of aliquot from 10⁻¹ dilution into empty plate 1, 1 mL of aliquot from 10⁻² dilution into empty plate 2, and 1 mL of aliquot from 10⁻³ dilution into empty plate 3.
- Open the lid/cotton plug of the flask with HSA media and flame its mouth. Pour the freshly prepared HSA media at the appropriate temperature.
- As discussed above in Section 5.2, pour the moderately hot liquid HSA media into the petri plates with inoculum and mix the media with the inoculum completely by gently swirling the plates clock-wise and counter clock-wise.
- Close the lid of the plate and allow the media to solidify.
- Incubate the plates in an inverted position at 35°C for 24–48 hours in an incubator.
- After incubation, check for the presence of purple-coloured colonies on the surface and sub-surface of the media, which are produced by *Salmonella* spp. The presence of these purple-coloured colonies confirms the presence of *Salmonella* in the sample, which can be enumerated and quantified by the procedure given in Section 7 below.

6.3.2 Spread plate method

- If media plates are kept in a refrigerator after autoclaving, bring all plates to room temperature (RT) before inoculation by keeping them outside at RT for 1–1.5 hours.
- Label media name, sample ID, sample dilution (e.g. 10^{-1} , 10^{-2} , and 10^{-3}) and type of plate-count technique used (spread plate) on the base of each petri plate with HSA media to be inoculated.
- Sterilize the glass spreaders using alcohol and flame. Dip the curved end of the spreader completely in 70% ethanol, remove excess ethanol, and sterilize by flaming in a Bunsen burner. Allow it to cool down. The glass spreader should be sterilized after every use.
- Add 0.1 mL of aliquot from each of 3 selected dilutions into separate petri dishes with HSA media (e.g. 1 mL of aliquot from 10^{-1} dilution into HSA plate 1, 1 mL of aliquot from 10^{-2} dilution into HSA plate 2, and 1 mL of aliquot from 10^{-3} dilution into HSA plate 3), and immediately spread (evenly) the inoculum by rotating the glass spreader in one direction and petri plate in the opposite direction so that the inoculum is evenly distributed throughout the plate. Spreading should be done until the inoculum is absorbed into the semi-solid media. Spreading allows the bacteria to disperse and separate throughout the media plate, so that they form isolated colonies for easy enumeration.
- After spreading is completed, let the plate sit for five to ten minutes before inverting and incubating them. Drying time varies with the room temperature and humidity.
- Close the lid and incubate the plates in an inverted position at 35°C for 24–48 hours in an incubator.
- After incubation, check for the presence of purple-coloured colonies on the surface of the media, which are produced by *Salmonella* spp. The presence of these purple-coloured colonies confirms the presence of *Salmonella* in the sample, which can be enumerated and quantified by the procedure given in Section 7 as follows.

7. Data analysis and calculations

- The estimated density of *Salmonella*, based on the above results, is calculated in terms of CFU/ mL.
- Due to the variability in the solid content of the biosolid sample derived from faecal sludge, *Salmonella* results from these samples are expressed as CFU/g total solids (dry weight basis).
- CFU/g total solids (dry weight) is calculated in two steps:
Step 1: Calculation of CFU/mL (wet weight)
Step 2: Conversion to CFU/g total solids (dry weight)

7.1 Step 1: Calculation of CFU/mL (wet weight)

7.1.1 Result interpretation

- For the optimum colony count, the number of colonies should be less than 200 in a plate. Beyond this limit, the whole procedure must be repeated by inoculating with higher dilutions of the sample.
- If the colonies are fused or not properly isolated or if the whole plate is covered with a bacterial mat, then the colonies cannot be counted and the plate has to be rejected. This plate is reported as 'too numerous to count' (TNTC), and the process has to be repeated with higher sample dilutions to get optimum number of colonies.
- If optimum number of colonies (< 200) are seen in all the plates, count the total number of (purple) colonies present in each plate inoculated with different sample dilution separately, and note down the results. In this case, take colonies of 20–60 for calculation of final result. If replicates of a dilution are inoculated, take the average of the optimum number of colonies observed in replicate plates of the same dilution, and use it for calculation given below.
- As, only '0.1 mL' of inoculum is used per dilution in 'spread-plate method', the 'number of colonies' obtained above has to be multiplied by '10', followed by multiplication of 'dilution factor' which gives the 'CFU/mL (wet weight)' of *Salmonella* present in the sample tested.
- In case of pour-plate method, as '1 mL' of inoculum is used per dilution, multiplying the 'number of colonies' obtained above with the 'dilution factor' gives the amount of *Salmonella* in 'CFU/mL (wet weight)' of the sample tested.
- For spread-plate method: $\text{CFU/mL (wet weight)} = \text{Number of colonies} \times 10 \times \text{Dilution factor} \times 10^*$
- For pour-plate method: $\text{CFU/mL (wet weight)} = \text{Number of colonies} \times \text{Dilution factor} \times 10^*$
- *Note: Since FS biosolid samples were diluted in the homogenization step (Section 6.1), the dilution must be taken into account when calculating CFU / mL (wet weight). As a result, the 'CFU/mL (wet weight)' is multiplied by '10' to account for diluting the sample during the homogenization step.

7.1.2 Determination of total solids

- As the treated/ partially treated/ untreated FS biosolid samples show large variability in their solid content, the MPN is calculated on a dry weight basis.
- For calculation of sample results on a dry weight basis, total solids in the sample have to be determined as follows:
- Take the weight of an empty clean crucible or aluminium evaporating dish (W_1) and tare it.

- Add 10-30 g of the sample to be tested into the tared crucible or aluminium evaporating dish and note down the weight (W_2). This step should be performed immediately after weighing the sample for microbiological analysis.
- Dry the crucible or aluminium evaporating dish along with the sample overnight in hot-air oven at 103–105 °C.
- After drying, keep the sample in a desiccator and allow it to cool. Take the weight of the crucible with dried sample (W_3).
- Subtract the empty crucible weight (W_1) from the weight of the crucible with dried sample (W_3) to get the weight of dried sample alone (W_4).
- So, $W_4 = W_3 - W_1$
- Calculate the % dry weight of the sample as follows:
- % Dry weight = (weight of dried sample in g [W_4] / weight of sample in g [W_2]) \times 100
- Note: The drying oven should be kept in a hood or be vented. Significant laboratory contamination may result from drying a heavily contaminated sample.

For the conversion to CFU / g total solids (dry weight), we assume that, CFU / mL (wet weight) = CFU / g (wet weight).

So, CFU / g (dry weight) = [CFU / mL (wet weight) from Step 1 (Section 7.1)] / percent total solids expressed as a decimal

Note: As *Salmonella* in biosolids is reported in CFU/4g (dry solids) (as per the units of available standards for *Salmonella* in biosolids), the following calculation is given for conversion to CFU/4g (dry solids).

$$\text{CFU / 4 g (dry weight)} = \frac{[\text{CFU / mL (wet weight) from Step 1 (Section 7.1)}] \times 4}{\text{Per cent total solids expressed as a decimal}}$$

6.11 STANDARD OPERATING PROCEDURE FOR HELMINTH EGGS ENUMERATION

Scope and field of application

The prevalence of helminth infections in people living with basic water and sanitation in developing countries such as most of those in India is generally high. Helminths are multicellular parasites, but their eggs are microscopic, commonly known as intestinal worms. Helminths are worm-like organisms living in and feeding on living hosts. It absorbs nutrients from the host for their growth and

causes weakness and disease in the host. They survive in mammalian hosts for many years due to their ability to manipulate the host's immune response by secreting immunomodulatory products. Helminths that live inside the digestive tract are called intestinal parasites and the infection caused by helminths is known as helminthiasis. Parasitic worms (helminths) can be found in the human intestinal tract, urinary tract, or bloodstream. Due to the extreme hardness of the eggs of the roundworm, *Ascaris lumbricoides*, they are used in the sanitation field as a 'marker for the safe reuse of human waste'. Other commonly found helminths are *Trichuris trichiura*, *Taenia sp.*, and in areas with very sandy soils, *hookworm sp.* It is generally accepted that if any of the various waste treatments used are successful in inactivating *Ascaris* eggs, then all harmful bacteria and viruses should also be killed.

Principle

Helminth eggs are thought to adhere to sludge and soil particles, possibly as a result of charge interactions with or adsorption of eggs to the particles. Many FS samples are often contaminated with silica/sand particles, hence the use of ammonium bicarbonate as a wash solution. Laboratory testing for helminths is based on four main principles: washing, filtration, centrifugation, and flotation of the eggs to remove them from the various waste mediums.

- Ammonium bicarbonate is used as both a wash solution and to dissociate the eggs from the soil particles.
- Filtration, using 100 μ m and/or 20 μ m sieves is used to separate larger and smaller particles from the eggs both after washing and after flotation.
- Flotation, using a solution of zinc sulphate at a specific gravity of 1.30 is used to float eggs with a relative density of <1.3 out of the matter retained with them on the 20 μ m sieve.

Safety precautions

- Sterile work area to reduce contamination from airborne particles and aerosols
- Good personal hygiene
- Wash your hands before and after working
- Always wear gloves and a laboratory coat or plastic apron while processing the samples
- Always wipe your hands and work area with 70 percent ethanol

Apparatus

- Compound microscope with 10x and 40x objectives

- Bench-top centrifuge with a swing-out rotor that can spin a minimum of 8 x 15 ml plastic conical test tubes (Falcon tubes)
- Sink with a hose attached to tap for washing using high water pressure
- Weighing balance (scale for weights up to 200 gm and accurate to 2 decimal places)
- Magnetic stirrer and bar magnets
- Vortex mixer
- Hydrometer that can measure specific gravity between 1.2 to 1.4
- 100µm mesh stainless steel flat sieve, diameter 200 mm
- 20µm mesh stainless steel flat sieve, 200 mm
- 20µm mesh stainless steel flat sieve, 100 mm
- Plastic test tube racks to hold the 15 ml Falcon tubes
- Plastic 200 ml beakers
- Plastic 'hockey-stick' shaped stirring rods or 'L' rods
- Plastic 3 ml pipettes
- Gloves
- Applicator sticks and wooden tongue depressors
- Microscope slides (76 x 26 x 1.2 mm)
- Cover glasses 22 x 40 mm

Chemicals and reagent preparation

- Zinc sulphate ($ZnSO_4$)

$ZnSO_4$ (heptahydrate) is made up by dissolving 500g of the chemical in 880 ml de-ionized or distilled water.

Note: A hydrometer must be used to adjust the specific gravity to 1.3, use more chemicals if the specific gravity is too low, or add more water if it is more than 1.3.

- Ammonium bicarbonate (NH_4HCO_3)

Sample type and quantity

Sample type	Example	Quantity
Liquid	Treated water / Ground water	2L-5 L
Semi-liquid	Black water / untreated waste water	250 mL-1L
Semi-solid		50 mL
Solid	Composted sludge, Domestic waste & thick sludge	10-50 g
Dry	Dry Compost	10 g

Dissolve 119 gm of ammonium bicarbonate in 1lt de-ionized water (use a magnetic stirrer and bar magnet)—store in a glass jar.

- Tween 80
- 0.1N sulphuric acid (H_2SO_4)

Procedure

- Weigh 10–20 g of sample into a 200 ml plastic beaker, on a top-pan balance.
- Add 50–80 ml NH_4HCO_3 and using a magnetic stirring bar, mix on a magnetic stirrer for 20 minutes.
- Pour mixture over 100 μm sieve which fits on top of a 20 μm sieve (wet sieves with tap H_2O first).
- Rinse the beaker with tap H_2O and pour over sieves.
- Wash magnet and remove, wash 100 μm sieve well (using ‘hockey sticks’ or gloved hand) over 20 μm filter, checking bottom sieve for fluid build-up.
- Separate sieves and then rinse the 20 μm sieve well and wash the material to one side of the sieve.
- Rinse all material off the 20 μm filter into the original rinsed-out beaker.
- Pour beaker contents into 4 x 15 ml conical test tubes.
- Centrifuge at 3000 rpm in centrifuge with swing-out rotor for five minutes.
- Pour off supernatant: deposits left in four test tubes.
- Place test tubes in the rack with an applicator stick in each (as a stirring rod) and pipette in ZnSO_4 , 3 ml at a time, vortexing in between addition of the chemical, until tubes are filled to 14 ml mark.
- Centrifuge at 2000 rpm for five minutes.
- Pour supernatant flotation fluid over a smaller diameter 20 μm sieve. Wash out test tubes and keep one aside for reuse.
- Wash the material on the sieve well with tap water and rinse it down to one side of the sieve for collection. Using a 3 ml plastic pipette, transfer the material back into the test tube kept aside.
- Centrifuge at 3000 rpm for 5 minutes to obtain the final deposit and reconstitute in 1 ml sterile distilled water.
- Pour off supernatant water and pipette up the deposit, place it on a counting chamber (hemocytometer), place a 22 x 40 mm cover slip on top, and examine and count every helminth egg, classifying them as viable, potentially viable, or dead.
- Calculate the number of viable eggs per slide.

Calculation

$$\text{No. of viable eggs per L} = \frac{\left(\frac{\text{No. of viable eggs in a slide (E*)} \times 1000}{\text{Volume of sample taken in a slide}} \right)}{\text{Initial volume of sample taken for analysis (ml)}}$$

$$\text{No. of viable eggs per gm of dry weight} = \frac{\left(\frac{\text{No. of viable eggs in a slide (E*)} \times 1000}{\text{Volume of sample taken in a slide}} \right)}{\text{Dry weight of sample taken for analysis (g)}}$$

$$E^* = A + B + C + D$$

Whereas

A = Number of fertilized Ascaris eggs

B = Number of unfertilized Ascaris eggs

C = Number of decorticated Ascaris eggs

D = Number of other helminths eggs

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Annexure

MOST PROBABLE NUMBER (MPN) PROCEDURE USING 5-REPLICATE TUBES PER DILUTION (5-REPLICATE TUBE METHOD)

A1.1 Data analysis and calculations

- When more than three dilutions are used in a decimal series of dilutions, then select 3 significant dilutions from the total dilutions (sample volumes) tested based on the guidelines given in Section A1.1.1 and shown in Table A1.1 below and note down the number of tubes showing positive results (valid series/ combination of positives) in the selected three significant dilutions as shown in Table A1.1 below.
- Note down the ‘MPN Index/ ml’ by matching the selected (valid) series/ combination of positives with the same series on the MPN reference chart given in Table A1.2. This gives MPN/ ml for the decimal dilutions (1 mL, 0.1 mL, 0.01 mL) given in Table A1.2.
- When the series of decimal dilutions is different from that given in Table A1.2 (1 mL, 0.1 mL, 0.01 mL), select the MPN value from Table A1.2 for the combination of positive results (valid series) and calculate according to the following formula:
$$\text{MPN/ mL} = (\text{MPN index/ mL given in Table A1.2}) / V$$
, where, V = volume of sample portion at the lowest selected dilution (highest sample volume/ highest sample quantity).

A1.1.1 Selection of significant dilutions

When more than three dilutions are used in a decimal series of dilutions, use the following guidelines to select the three most appropriate dilutions:

- First, remove the highest dilution (smallest sample volume) if it has all negative tubes and at least one remaining dilution has a negative tube. Next, remove the lowest dilution (largest sample volume) if it has all positive tubes and at least one remaining dilution has a positive tube.
- According to these guidelines, the three dilutions in Example A in ‘Table A1.1’ are selected by removal of the highest (0.001-mL) and the lowest (10-mL) dilutions.
- If the lowest dilution does not have all positive tubes, and several of the highest dilutions have all negative tubes, then remove the highest negative dilutions (Example B in ‘Table A1.1’).

- More than three dilutions may remain after removal of the lowest dilution with all positive tubes and high dilutions with all negative tubes. In this case, if the highest dilution with all positive tubes is within two dilutions of the highest dilution with any positive tubes, then use the highest dilution with any positive tubes and the two immediately lower dilutions. In Example C in 'Table A1.1', the highest dilution with all positive tubes is 0.1 mL, which is within two dilutions of 0.001 mL, which has one positive tube.
- In Example D in 'Table A1.1', the highest dilution with all positive tubes is 0.01 mL, which is within two decimal dilutions of 0.001 mL, to yield a combination of 4-5-1. If, after removal of the lowest dilution with all positive tubes, no dilution with all positive reactions remains, then select the lowest two dilutions and assign the sum of any remaining dilutions to the third dilution.
- In Example E in 'Table A1.1', the highest dilution with all positive tubes contains 10 mL; this dilution was removed in the second step. Four dilutions, none of which have all positive tubes, remain. Under these circumstances, select the two lowest remaining dilutions corresponding to 1 and 0.1 mL sample. For the third dilution, add the number of positive tubes in all higher dilutions (0.01 and 0.001 mL sample), to yield a final combination of 4-4-1.
- If no dilution has all positive tubes (Example F in 'Table A1.1'), select the lowest two dilutions, corresponding to 10 and 1 mL sample. For the third dilution, add the number of positive tubes in the remaining dilutions (0.1, 0.01, and 0.001 mL sample), to yield a final combination of 4-3-2.
- If the third dilution is assigned more than five positive tubes, then the selected combination will not be in Table A1.1. If the three dilutions selected are not found in Table A1.1, then something in the serial dilution was unusual. In this case, the usual methods for calculating the MPN, presented here, may not apply. If a new sample cannot be collected and an MPN value is still desired, use the highest dilution with at least one positive tube and the two dilutions immediately lower as the three selected dilutions. In Example G in 'Table A1.1', the first selection, 4-3-6 (the outcome from the highest three dilutions), is not in Table A1.1 because 6 is greater than 5. The second selection, according to the above guidelines, would be 3-2-1.
- For additional guidelines on the selection of significant dilutions, refer to 'US EPA Method 1680/Method 1681'.
- For further details on the calculation of MPN, refer to 'APHA (2023): Standards methods for the examination of water and wastewater, 9221 C'

Table A1.1: Examples for choice of three combinations of positives from five dilutions

Example	Volume mL					Combination of Positives	MPN Index No./100 mL
	10	1	0.1	0.01	0.001		
A	5	5	1	0	0	x-5-1-0-x	330
B	4	5	1	0	0	4-5-1-x-x	48
C	5	2	5	2	1	x-x-5-2-1	7000
D	4	5	4	5	1	x-x-4-5-1	4800
E	5	4	4	0	1	x-4-4-1-x	400
F	4	3	0	1	1	4-3-2-x-x	39
G	4	3	3	2	1	x-x-3-2-1	1700

Table A1.2 MPN Index and 95% confidence limits for various combinations of positive results when five tubes are used per dilution (1.0 mL, 0.1 mL, 0.01 mL)* (5-replicate tube method)

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<0.1803			1-3-0	0.83	0.12	1.96
0-0-1	0.18	0.03	0.63	1-3-1	1.04	0.20	2.43
0-0-2	0.36	0.03	1.01	1-3-2	1.25	0.29	2.96
0-0-3	0.54	0.03	1.37	1-3-3	1.47	0.38	3.64
0-0-4	0.72	0.08	1.74	1-3-4	1.69	0.48	4.60
0-0-5	0.91	0.15	2.12	1-3-5	1.91	0.57	5.66
0-1-0	0.18	0.03	0.63	1-4-0	1.05	0.21	2.45
0-1-1	0.36	0.03	1.01	1-4-1	1.27	0.30	3.00
0-1-2	0.55	0.03	1.38	1-4-2	1.48	0.39	3.70
0-1-3	0.73	0.08	1.75	1-4-3	1.70	0.48	4.68
0-1-4	0.91	0.15	2.14	1-4-4	1.93	0.58	5.75
0-1-5	1.10	0.23	2.56	1-4-5	2.15	0.67	6.57
0-2-0	0.37	0.03	1.02	1-5-0	1.28	0.30	3.03
0-2-1	0.55	0.03	1.39	1-5-1	1.50	0.40	3.75
0-2-2	0.74	0.08	1.76	1-5-2	1.72	0.49	4.77
0-2-3	0.92	0.15	2.15	1-5-3	1.95	0.58	5.83
0-2-4	1.11	0.23	2.58	1-5-4	2.17	0.68	6.64
0-2-5	1.29	0.31	3.07	1-5-5	2.40	0.77	7.31
0-3-0	0.56	0.03	1.40	2-0-0	0.45	0.03	1.19
0-3-1	0.74	0.09	1.77	2-0-1	0.68	0.06	1.64
0-3-2	0.93	0.16	2.17	2-0-2	0.91	0.15	2.13
0-3-3	1.12	0.23	2.60	2-0-3	1.15	0.25	2.69
0-3-4	1.30	0.31	3.10	2-0-4	1.39	0.35	3.38
0-3-5	1.49	0.39	3.72	2-0-5	1.64	0.46	4.37
0-4-0	0.75	0.09	1.79	2-1-0	0.68	0.06	1.66
0-4-1	0.94	0.16	2.19	2-1-1	0.92	0.15	2.16

STANDARD OPERATING PROCEDURE FOR TESTING WASTEWATER, FAECAL SLUDGE AND BIOSOLIDS

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-4-2	1.12	0.24	2.63	2-1-2	1.16	0.25	2.72
0-4-3	1.31	0.32	3.13	2-1-3	1.41	0.36	3.43
0-4-4	1.50	0.40	3.77	2-1-4	1.66	0.46	4.47
0-4-5	1.69	0.48	4.62	2-1-5	1.92	0.57	5.71
0-5-0	0.94	0.16	2.21	2-2-0	0.93	0.16	2.18
0-5-1	1.13	0.24	2.65	2-2-1	1.18	0.26	2.76
0-5-2	1.33	0.32	3.17	2-2-2	1.43	0.36	3.49
0-5-3	1.52	0.40	3.82	2-2-3	1.68	0.47	4.56
0-5-4	1.71	0.48	4.70	2-2-4	1.94	0.58	5.81
0-5-5	1.90	0.56	5.63	2-2-5	2.21	0.69	6.75
1-0-0	0.20	0.03	0.68	2-3-0	1.19	0.26	2.79
1-0-1	0.40	0.03	1.08	2-3-1	1.44	0.37	3.55
1-0-2	0.60	0.03	1.49	2-3-2	1.70	0.48	4.67
1-0-3	0.81	0.11	1.91	2-3-3	1.97	0.59	5.91
1-0-4	1.01	0.19	2.36	2-3-4	2.23	0.70	6.83
1-0-5	1.22	0.28	2.87	2-3-5	2.51	0.82	7.59
1-1-0	0.40	0.03	1.09	2-4-0	1.46	0.38	3.61
1-1-1	0.61	0.03	1.50	2-4-1	1.72	0.49	4.77
1-1-2	0.81	0.11	1.92	2-4-2	1.99	0.60	6.00
1-1-3	1.02	0.19	2.38	2-4-3	2.26	0.72	6.92
1-1-4	1.23	0.28	2.90	2-4-4	2.54	0.83	7.68
1-1-5	1.44	0.37	3.54	2-4-5	2.82	0.94	8.36
1-2-0	0.61	0.03	1.51	2-5-0	1.74	0.50	4.88
1-2-1	0.82	0.12	1.94	2-5-1	2.01	0.61	6.10
1-2-2	1.03	0.20	2.40	2-5-2	2.29	0.73	7.00
1-2-3	1.24	0.29	2.93	2-5-3	2.57	0.84	7.76
1-2-4	1.46	0.38	3.59	2-5-4	2.86	0.95	8.45
1-2-5	1.67	0.47	4.51	2-5-5	3.15	1.07	9.10
3-0-0	0.79	0.10	1.88	4-3-0	2.71	0.90	8.09
3-0-1	1.06	0.21	2.46	4-3-1	3.26	1.11	9.34
3-0-2	1.35	0.33	3.23	4-3-2	3.86	1.32	10.60
3-0-3	1.65	0.46	4.40	4-3-3	4.51	1.54	11.92
3-0-4	1.96	0.59	5.89	4-3-4	5.21	1.76	13.31
3-0-5	2.29	0.73	6.99	4-3-5	5.93	1.96	14.77
3-1-0	1.07	0.22	2.50	4-4-0	3.35	1.14	9.53
3-1-1	1.37	0.34	3.29	4-4-1	3.98	1.37	10.84
3-1-2	1.67	0.47	4.52	4-4-2	4.66	1.59	12.23
3-1-3	1.99	0.60	6.01	4-4-3	5.39	1.81	13.68
3-1-4	2.32	0.74	7.10	4-4-4	6.15	2.02	15.21
3-1-5	2.67	0.88	8.00	4-4-5	6.93	2.23	16.81

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
3-2-0	1.38	0.35	3.35	4-5-0	4.11	1.41	11.11
3-2-1	1.70	0.48	4.64	4-5-1	4.83	1.64	12.56
3-2-2	2.02	0.62	6.13	4-5-2	5.59	1.87	14.09
3-2-3	2.36	0.76	7.20	4-5-3	6.39	2.09	15.70
3-2-4	2.71	0.90	8.10	4-5-4	7.22	2.30	17.39
3-2-5	3.08	1.04	8.94	4-5-5	8.06	2.50	19.16
3-3-0	1.72	0.49	4.77	5-0-0	2.40	0.76	7.63
3-3-1	2.05	0.63	6.24	5-0-1	3.14	1.06	9.08
3-3-2	2.40	0.77	7.31	5-0-2	4.27	1.46	11.42
3-3-3	2.76	0.92	8.21	5-0-3	5.78	1.92	14.46
3-3-4	3.13	1.06	9.06	5-0-4	7.59	2.39	18.16
3-3-5	3.52	1.20	9.89	5-0-5	9.53	1.65	22.34
3-4-0	2.09	0.64	6.36	5-1-0	3.29	1.12	9.40
3-4-1	2.44	0.79	7.42	5-1-1	4.56	1.56	12.02
3-4-2	2.81	0.93	8.33	5-1-2	6.31	2.07	15.53
3-4-3	3.19	1.08	9.18	5-1-3	8.39	2.57	19.85
3-4-4	3.58	1.23	10.02	5-1-4	10.62	3.04	24.85
3-4-5	3.99	1.37	10.86	5-1-5	12.93	3.04	30.90
3-5-0	2.48	0.80	7.53	5-2-0	4.93	1.67	12.76
3-5-1	2.86	0.95	8.44	5-2-1	7.00	2.24	16.94
3-5-2	3.25	1.10	9.31	5-2-2	9.44	2.80	22.13
3-5-3	3.65	1.25	10.17	5-2-3	12.05	3.31	28.43
3-5-4	4.07	1.40	11.03	5-2-4	14.79	3.81	37.14
3-5-5	4.50	1.54	11.89	5-2-5	17.67	5.03	52.30
4-0-0	1.30	0.31	3.11	5-3-0	7.92	2.47	18.86
4-0-1	1.66	0.46	4.45	5-3-1	10.86	3.08	25.44
4-0-2	2.07	0.64	6.31	5-3-2	14.06	3.68	34.45
4-0-3	2.53	0.82	7.64	5-3-3	17.50	4.34	51.31
4-0-4	3.02	1.02	8.81	5-3-4	21.22	5.29	67.98
4-0-5	3.55	1.21	9.96	5-3-5	25.27	8.14	79.71
4-1-0	1.69	0.48	4.60	5-4-0	12.99	3.48	31.08
4-1-1	2.12	0.66	6.46	5-4-1	17.24	4.29	49.75
4-1-2	2.58	0.85	7.79	5-4-2	22.12	5.63	70.87
4-1-3	3.10	1.05	8.98	5-4-3	27.81	8.82	86.00
4-1-4	3.65	1.25	10.16	5-4-4	34.54	11.59	101.10
4-1-5	4.25	1.45	11.38	5-4-5	42.56	14.37	118.00
4-2-0	2.16	0.67	6.61	5-5-0	23.98	7.62	76.29
4-2-1	2.64	0.87	7.94	5-5-1	34.77	11.72	101.60
4-2-2	3.17	1.08	9.15	5-5-2	54.22	17.91	141.90
4-2-3	3.75	1.29	10.37	5-5-3	91.78	26.72	220.10

Combination of Positives	MPN Index mL	95% Confidence Limits		Combination of Positives	MPN Index mL	95% Confidence Limits	
		Lower	Upper			Lower	Upper
4-2-4	4.38	1.50	11.64	5-5-4	160.90	38.37	410.30
4-2-5	5.04	1.71	12.97	5-5-5	>160.90		

*Table A1.2 was developed using the MPN calculator developed by Albert Klee (1993).

*Results to two significant figures



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