M.Sc. ZOOLOGY LAB MANUAL

3rd Semester

Prepared By

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Zoology

MIDNAPORE CITY COLLEGE

PREFACE TO THE FIRST EDITION

This is the first edition of the Lab Manual the for PG Zoology Shird Semester. Hope this edition will help you during practical class. This edition mainly tried to cover the whole syllabus. Some hardcore instrument-based topics are not included here that will be guided by respective teachers at the time of the practical.

ACKNOWLEDGEMENT

We are really thankful to our students, teachers, and non-teaching staffs to make this effort a little bit complete. Mainly thanks to Director Sirs and Principal Sir and Teacher-in-charge Sir to motivate for making this lab manual.

Paper: ZOO 395: Entomology, Ecotoxicology, Molecular Evolution and Microbiology (Practical)

1) Entomology

a) Method of collection and preservation of insects

b) Study of the behavioural modification of legs in honey bee.

c) Entomological comments on common Pests, Aquatic insects, Insects of medical and economical importance. Galls & Seed cocoon

d) Mounting of sting apparatus & coupling device of Honey bee.

2) Ecotoxicology

a) Dose response curve

b) Lethal dose estimation

c) MATC in a fish species

d) Lethal dose50 mortality curve

3) Molecular Evolution

4) Microbiology

a. Staining and identification of bacteria, endospores, etc. from a culture media.

b. Different methods of staining: Gram staining, Negative and differential staining.

c. Preparation of different culture media with Sterilization techniques.

d. Inoculation of microbes to respective culture media through proper culture methods.

e. Enumeration of Coliform bacteria using multiple tube fermentation method.

Special Paper Practical

Course No: ZOO 396A: Fishery Practical-I (50 Marks, 4 credit)

Syllabus:

1. Identification of fin fish, ray fish, and shellfish.

2. Qualitative and Quantitative estimation of zooplankton.

3. Identification of fish parasites.

4. ARO in fishes.

5. Identification of shellfishes.

6. Identification of different development stages of prawns and crabs.

7. Visit shrimp, prawn, and biofloc hatcheries.

8. Field study.

Course No: ZOO 396B: Ecology Practical-I (50 Marks, 4 credit)

Syllabus:

1. Preparation of Climograph

2. Estimation of transparency, TSS, TDS, conductivity, hardness, salinity and alkalinity of water.

3. Estimation of N, P, and K content of water/ soil.

4. Basic principle pertaining to acid digestion for the estimation of heavy metals in the water sample.

5. Ecological comments on major biotic components in the Aquatic system

6. Recording/documentation and submission of terrestrial/aquatic faunal components in and local areas – (Collection, preservation, identification and analysis of aquatic biota – phytoplankton, zooplankton, benthos, periphyton, aquatic insects, nekton and macrophytes).

7. Applicability of GPS/GIS in recording bioresources and mapping the landscape.

8. Submission of Laboratory notebook.

9. Field study

Course No: ZOO 396C: Genetics & Molecular Biology Practical-I

(50 Marks, 4 credit)

Syllabus:

1. Preparation of mitotic metaphase chromosome of the rat.

2. Bacterial Genomic and Plasmid DNA isolation and restriction digestion and agarose gel electrophoresis.

3. Genomic DNA isolation from blood and restriction digestion

4. Sophisticated Laboratory /Research Institute/ University visit

ZOO 395: Entomology

Methods of Collection of Insects

Techniques for collecting insects are varied. However when collecting insects, we should remember/ keep in mind the following:

a) The requirements for collecting aquatic insects from rivers, streams, vegetation etc, would differ from those for collecting terrestrial insects from trees, soil, debris, etc.

b) The collecting method does not only depend on the insect type or group to be collected, but it also varies with the need of collecting live or dead insects.

c) The collecting method being chosen and the collecting supplies required also varies with the time of the day, collection would be carried out, thus, requirements vary for collection during the day from that at night.

Categorising the Methods of Collection:

The methods used for collecting insects may be divided into two categories – Active and Passive. In the first category, the collector is involved in an active search in finding out the insects either by hand or by using the apparatus suited according to one's needs. In the second category, the traps of different kinds are employed to do the work, and the collector participates only passively.

Active Collecting Methods:

Hand Collecting

When insects are collected by hand then one should have good knowledge or proper information as to which insects bite and which sting, and how far such attack can prove to be poisonous. Caterpillars with spines or hairs on their bodies should be avoided because some of these have venom associated with these structures. In case of collection by hand, the collector should be well equipped with tools like – hatchet, knife, small garden shovel, forceps, brush, gloves hand-lens, pocket knife, etc.

Hand collecting is useful for large but sedentary or slow-moving insects. Insects found in places such as under logs or rocks/ stones or loose bark or in buildings, in crevices or beddings or timber, or in dung, or in different parts of the plants or in any other location where using any larger equipment is not possible, must be collected by hand.

Aspirators

Aspirators also known as 'pooters' are effectively used to capture small insect specimens in a convenient way. Insects that rarely fly might also be easily collected using this device. It consists of a glass or Perspex (plastic) or celluloid vial/ jar/ bottle or test tube as required, of the desired diameter and length, fitted or sealed with a cover; two pieces of rigid tubing of either glass of metal or plastic, one short and the other long ; a cover, provided with two holes in which the tubing will fit properly, and a piece of flexible tubing of either rubber or plastic, with a diameter just large enough to fit tightly over one end of shorter piece of the rigid tubing.

Beating

Wingless and/ or non-flying ad slow-moving insects can be easily collected using a beating sheet/ tray. Well camouflaged or hidden species of insects resting/ securely perched on bushes/ shrubs/ trees, thus, those difficult to be easily spotted, might be conveniently collected through this technique.

Sifters

Sifters are used for collecting concentrated quantities of litter and soil, containing many insect species. Sifters might be of different kinds. One kind has two hoops of heavy metal, each with a handle.

Separators and Extractors

A modification of the sifter is a separator. These devices usually depend on some physical aid such as light, heat, or dryness to impel the insects to leave the foreign matter. The simplest of such devices is the sweeping separator.

Soil Washing/ Floatation Samplers

To collect the insects in arid/ deep/ mineral soils with high clay content, this is an effective technique. Soil washing requires the ratio of water to soil to be about 4:1. This technique provides the complete life-history data of the insects collected.

Vacuum Sampling

Widely used power suction samples include "D-vac" and "McCoy Insect Collector", are used to collect insects through vacuum sampling.

Pupa Digging

This method requires a trowel, for digging and to lift the material containing the specimens. The insects are ultimately sorted out on a water proof white sheet.

Nets

The net is a very handy tool and considered a much valuable equipment for collecting insects, of the three commonly available insect nets, i.e., aerial or butterfly net, sweeping net and the aquatic net.

Type of Nets:

Aerial/ Butterfly Net: It is designed to collect specific groups of insects, namely – dragonflies, butterflies, moths, bees, wasps, flies, some smaller insect varieties, etc. The collecting bag is usually white in colour.

Sweep Net/ Beating Net: It is made up of much heavier, sturdier and a more durable material such as canvas or thick cotton cloth or strong muslin. This net is used to collect grasshoppers, bugs, beetles and chewing and sucking insects of varied sizes.

Passive Collecting Methods:

Traps

Anything that impedes or stops the progress of an insect is referred to as a 'trap'. The factors affecting the performance of a trap include construction of the trap, location of the trap, time of the year or day chosen for trapping, weather and temperature on the day of trapping, kind of the attractant used, if any.

Funnel Trap

The trap consists of an electric light source, with a funnel at the bottom directed either into a killing jar or a dark box, positioned below the funnel, to prevent the escape of any insects.

Pan Trap/ Yellow-Pan Trap/ Moericke Trap: In this trap, small, shallow pans, mostly yellow in colour filled with a liquid (water mixed with a surfactant), is used to trap insects.

Coloured object/ Sticky Trap: In this type of trap a piece of tape, simple plastic disposable drinking cups, a cylinder, board, cards, a pane of glass, a piece of wire net is required.The object is usually painted yellow, attracting diverse groups of insects, and coated with a sticky substance such as 'Flytac', Tangle foot etc.

Yellow Sticky Strip: Usually 11.5 inches X 6 inches, thin strips made up of plastic, bright yellow in colour and coated on both sides with a non-toxic, non-drying sticky substance. These strips are covered on either side with a removable waxed-paper.

Pitfall Trap

Pitfall traps are another kind of trap employed by the Bombay Natural History Society, for collecting insects. A pitfall trap consists of some type of cup or container like plastic buckets, jam tins, glass jars, can, dish are buried into the ground in a manner that the upper rim or lip or the top edge of the container, flushes with the ground/ soil surface.

Flight - Interception Trap (FIT)

Also known as barrier trap, this trap is used to collect small, weak flying species of insects; those fly upwards or fall downwards on being intercepted by a barrier into a container of some type, which is partially filled with a suitable liquid preservative/ killing agent.

Suction and Rotary Trap

These traps either pump a volume of air through a filter (Johnson, 1950) or use a mechanically rotated net, to obtain aerial insect fauna. Suction traps collect small slow-moving, fragile, winged insect specimens.

Electrical Grid Trap

In this kind of trap, insects are attracted to pheromone or any other kind of attractant which is placed in a chamber, and protected by a strongly charged electrical grid, hence, trapped.

Light

The fact that insects are attracted to light from any source is used to trap insects, especially those which are nocturnal in habit. Torch lights and lanterns are use to catch nocturnal beetles and moths.

Collecting Aquatic Insects:

When collecting aquatic insects, a general observation is that a sunny day, with the outside temperatures on the higher side, provides the collector with good results as both adults and the immature stages of aquatic insects get trapped. Different collecting equipment include –

NET: To collect insects many kinds of net might be used depending on the portion of the aquatic habitat the collector wishes to scoop. Water nets must be sturdy in nature, but the rims should be of a diameter smaller than those used for collecting aerial or terrestrial insects.

Underwater Light Trap: A light trap is used in under water expeditions to capture the water bugs, the water beetles and the aquatic stages (nymphs and larvae) of the may flies, dragon flies and various other flies (Diptera).

KILLING AGENTS

Killing jars: used for terrestrial insects. Consist of a glass jar with a thin layer of plaster-ofParis in the bottom. The plaster layer is saturated with ethyl acetate and the insects placed in the jar are killed by asphyxiation.
Ethanol: aquatic and soft bodied insects may be preserved in 70% Ethanol.

Equipment and methods for preserving and mounting insects:
PINNING

Direct pinning: Pin is inserted directly through the body of the specimen. Pins are available in sizes from 000 to 7 but generally the insect is pinned with the thickest one it will take without damage. The specimen should be pinned through the thorax or elytra (see figures) and arranged so the appendages are well displayed for study. Use the first level of the pinning block to obtain the correct level of the insect on the pin. Sufficient pin must be left exposed above the specimen to allow safe handling, so large, robust insects may have to be mounted by eye, rather than on the pinning block.

Pointing: Insects that are too small to be pinned directly may be mounted on a point. A point is a triangular piece of stout white paper that you can make with a point punch. A pin is inserted through the broad end of the point and the small end is bent with forceps at a right angle. A very small amount of glue is placed on this turned-over tip and this is then applied to the right side of the thorax of the insect (see figures). A correctly pointed specimen has its body horizontal when the pin is upright, with the long axis of the body at right angles to the point.

Gluing: Small Diptera (true flies) are mounted by applying a small amount of glue (clear finger nail polish) about below the top of the pin and then touching it to the right side of the thorax of the insect.

PRESERVATION IN LIQUID: Most larvae and nymphs (immature insects) and some adult insects that are soft-bodied, must be preserved in liquid rather than pinned. It is best to kill the specimen in boiling water prior to preservation as this leaves them plump and limp and deactivates autolytic enzymes. A number of preservatives can be used but a solution of 70% alcohol, which preserves specimens in a supple condition, is one of the best. The specimens are stored in a stoppered vial with the data labels enclosed.

SLIDES: Many techniques have been developed for slide-mounting insects that are too small for pinning. Arrange object on slide so position suitable for study and customary for objects of its nature, that appendages or other structures are spread out and displayed. Various mounting media, some require dehydration and clearing of the specimen before mounting.

NOTE: VIALS AND SLIDES SHOULD NOT BE STORED IN THE SAME BOX AS DRY SPECIMENS

SPREADING: The wings of many insects show important taxonomic characters and should be mounted in a manner that will allow their examination. If the wings do not spread naturally when pinned, they must be spread manually. Spreading is done using a special board, called a spreading board. The spreading board has a central groove for the insect's body and a surface on either side of the groove on which the wings are pinned. Fresh, pliable specimens must be used (older specimens can be relaxed and then pinned). The wings are manipulated by inserting the point of a very fine pin (000 preferably) behind a main longitudinal vein. In most insects the fore wings are pushed forward until their posterior margins are in line with each other and at right angles to the body. Next, the hind wings are brought forward in the same manner until the anterior margin of each is just underneath the posterior margin of the fore wing. The wings are held in place by pins, or by strips of paper held down by pins. The abdomen and antennae may need to be supported by pins to prevent drooping. Larger specimens should remain on the board for 3 weeks to ensure complete drying. Spreading board (From Elzinga 2000)

Labelling collected insect specimens: An unlabeled specimen is incomplete and unacceptable. Labels should not be larger than 6x16 mm. Labels should be written in pencil, or computer-generated. Every specimen must have the following information on the label:

1st line – Specimen number and order
2nd line - Place of collection (country/ state and county)
3rd line - Place of collection (nearest post office)
4th line - Date collection
5th line - Name of collector

Study of the Behavioural Modifications in the Legs of Honey Bee

Systematic Position of Honey Bee:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta
Subclass: Pterygota
Order: Hymenoptera
Genus: Apis
Species: *Apis cerena indica*

Scientific Name: *Apis cerena indica*

There are three steps of leg modifications in three pairs of legs. For various types of activity different part of legs are modified in different manner in honey bee.

Limbs	Specialization	Position
Fore-limb	Antenna cleaner	Near the joint of tibia and tarsus
Middle-limb	Tibia spur	On distant of tibia
Hind-limb	Pollen basket	First tarsal segment

Economically Important Insects

Honey Bee:

Systematic Position:

Phylum: Arthropoda
Subphylum: Mandibulata
Class: Insecta
Subclass: Pterygota
Order: Hymenoptera
Genus: Apis
Species: *Apis cerena indica*

Importance:

Honeybees are of huge economic importance, vital for the pollination of many fruit, vegetable and seed crops. Also a wide variety of important products are made from the honey, beeswax, pollen, royal jelly and propolis that bees produce. It is the world's most important pollinator of food crops. It is estimated that one third of the food that we consume each day relies on pollination mainly by bees, but also by other insects, birds and bats.

Leptocorisa sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Hemiptera

Genus: Leptocorisa

Species: *L. acuta / L. varicornis*

Importance:

Rice bugs feed by inserting their needlelike mouthparts into new leaves, tender stems and developing grains. Excessive feeding can cause yellow spots on the leaves. This reduces photosynthesis and, in extreme cases, can damage the vascular system of the plant. Puncture holes also serve as points of entry for several plant pathogens, such as the fungus that causes sheath rot disease. The most economically important damage is caused when the adults and nymphs

feed on the developing grains. Such damage causes discoloration of the grains, which reduces market quality.

Scirpophaga sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Lepidoptera

Genus: Scirpophaga

Species: *S. incertulus*

Importance:

This moth is a major pest of paddy in India. Only the caterpillars are destructive which bores tunnel into the stem of the paddy plant. The plants attacked in early stages produces ears devoid of grain known as white ear.

Hispa sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Coleoptera

Genus: Hispa

Species: *H. armigera*

Importance:

It is a major pest of paddy particularly in north India. Both adult and larva causes damage to the crop.

Apion sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Coleoptera

Genus: Apion

Species: *A. corchori*

Importance:

Adult female bores a hole in the jute stem generally in the apical region and particularly at the base of the petioles for oviposition, thus, adversely affects the development of fibres. Hatched out larvae feeds on surrounding tissue causing extensive damage to the fibre crop.

Sitophilus sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Coleoptera

Genus: Sitophilus

Species: *S. oryzae*

Importance:

This beetle causes heavy damage to rice, wheat, and sorghum particularly in monsoon. This pest of stored grain also attacks oats, barley and cotton seeds. These weevils destroy more than they feed on.

Bombyx sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Lepidoptera

Genus: Bombyx

Species: *B. mori*

Importance:

The secretion of silk gland after coming into contact of air, harden to produce shiny silken thread to be used for formation of cocoon. This thread called as silk is a natural fibre and is of immense importance. Silk is used for:

- For making expensive garments,
- For certain industrial and electrical items.

Lac Insect:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

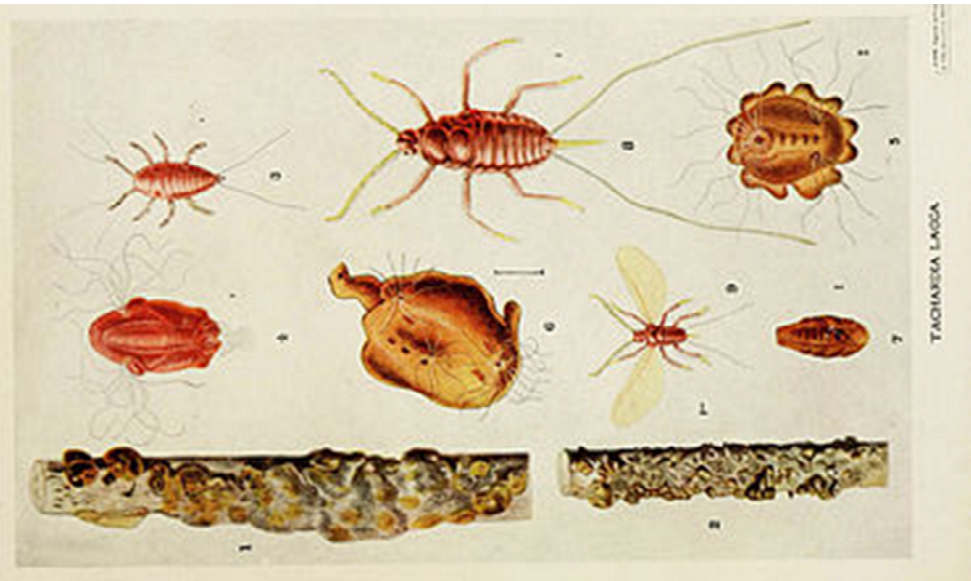
Class: Insecta

Subclass: Pterygota

Order: Hemiptera

Genus: Tachardia

Species: *T. lacca*



Importance:

Lac insect produces lac which is of commercial importance in both dosmestic as well as industrial sectors. It is used for the manufacture of :

-Varnishes and polishes
-Lithographic ink
-Sealing wax
-Electric insulation material
-Photographic materials
-Toys
-Jewellery items

Anopheles sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

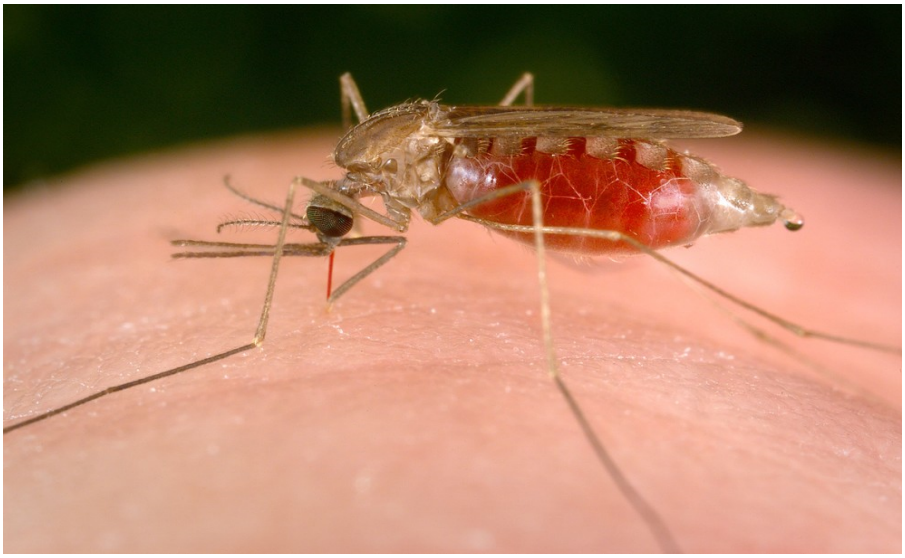
Class: Insecta

Subclass: Pterygota

Order: Diptera

Genus: Anopheles

Species: *Anopheles sp.*



Importance:

It acts as a vector of human malarial parasite belonging to the genus Plasmodium. Only female acts as a vector. In West Bengal, *Anopheles stephensi* has become major anopheline species responsible for transmission of malaria.

Culex sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Diptera

Genus: Culex

Species: *Culex sp*.

Importance:

Female Culex mosquito acts a vector of filarial worm, the microfilariae is transferred to human during blood meal.

Aedes sp.:

Systematic Position:

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Diptera

Genus: Aedes

Species: *Aedes sp.*

Importance:

Female Aedes can harbor viruses of diseases like dengue and Chicken guinea and thereby spreads the disease.

Cocoon :

A cocoon is a casing spun of silk by many moths and caterpillars, and numerous other holometabolous insect larvae as a protective covering for the pupa.

Cocoons may be tough or soft, opaque or translucent, solid or meshlike, of various colors, or composed of multiple layers, depending on the type of insect larva producing it. Many moth caterpillars shed the larval hairs (setae) and incorporate them into the cocoon; if these are urticating hairs then the cocoon is also irritating to the touch. Some larvae attach small twigs, fecal pellets or pieces of vegetation to the outside of their cocoon in an attempt to disguise it from predators. Others spin their cocoon in a concealed location—on the underside of a leaf, in a crevice, down near the base of a tree trunk, suspended from a twig or concealed in the leaf litter.

The silk in the cocoon of the silk moth can be unraveled to harvest silk fibre which makes this moth the most economically important of all lepidopterans. The silk moth is the only completely domesticated lepidopteran and does not exist in the wild.

Insects that pupate in a cocoon must escape from it, and they do this either by the pupa cutting its way out, or by secreting enzymes, sometimes called cocoonase, that soften the cocoon. Some cocoons are constructed with built-in lines of weakness along which they will tear easily from inside, or with exit holes that only allow a one-way passage out; such features facilitate the escape of the adult insect after it emerges from the pupal skin.

The cocoon is made of a thread of raw silk from 300 to about 900 m (1,000 to 3,000 ft) long. The fibers are very fine and lustrous, about 10 μm (0.0004 in) in diameter. About 2,000 to 3,000 cocoons are required to make one pound of silk (0.4 kg). At least 70 million pounds of raw silk are produced each year, requiring nearly 10 billion cocoons.

Leaf Gall :

Galls (from Latin galla, 'oak-apple') or cecidia (from Greek kēkidion, anything gushing out) are a kind of swelling growth on the external tissues of plants, fungi, or animals. Plant galls are abnormal outgrowths of plant tissues, similar to benign tumors or warts in animals. They can be caused by various parasites, from viruses, fungi and bacteria, to other plants, insects and mites. Plant galls are often highly organized structures so that the cause of the gall can often be determined without the actual agent being identified. This applies particularly to some insect and mite plant galls. The study of plant galls is known as cecidology.In human pathology, a gall is a raised sore on the skin, usually caused by chafing or rubbing.

Insect galls are the highly distinctive plant structures formed by some herbivorous insects as their own microhabitats. They are plant tissue which is controlled by the insect. Galls act as both the habitat and food source for the maker of the gall. The interior of a gall can contain edible nutritious starch and other tissues. Some galls act as "physiologic sinks", concentrating resources in the gall from the surrounding plant parts. Galls may also provide the insect with physical protection from predators.

Insect galls are usually induced by chemicals injected by the larvae of the insects into the plants, and possibly mechanical damage. After the galls are formed, the larvae develop inside until fully grown, when they leave. In order to form galls, the insects must take advantage of the time when plant cell division occurs quickly: the growing season, usually spring in temperate climates, but which is extended in the tropics.

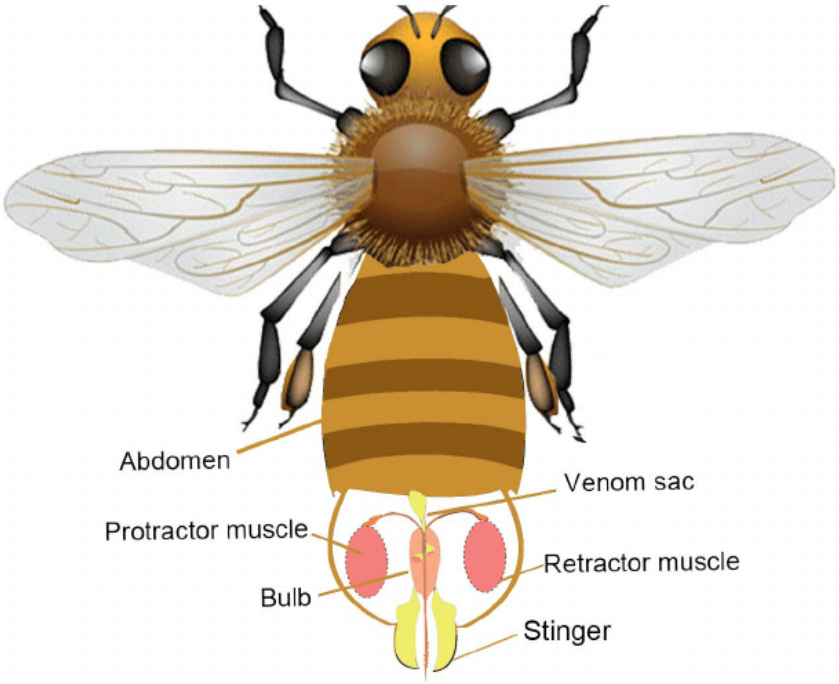
The meristems, where plant cell division occurs, are the usual sites of galls, though insect galls can be found on other parts of the plant, such as the leaves, stalks, branches, buds, roots, and even flowers and fruits. Gall-inducing insects are usually species-specific and sometimes tissue-specific on the plants they gall.

Galls are rich in resins and tannic acid and have been used in the manufacture of permanent inks (such as iron gall ink) and astringent ointments, in dyeing, and in tanning. A high-quality ink has long been made from the Aleppo gall, found on oaks in the Middle East. The Talmud records using gallnuts as part of the tanning process as well as a dye-base for ink.

The larvae in galls are useful for a survival food and fishing bait; see the Indigenous Australian foods Bush coconut and Mulga apple. Nutgalls also produce purpurogallin.

The gall of Rhuschinensis, Gallachinensi, has long been considered to possess many medicinal properties.

Sting apparatus of Honey bee.

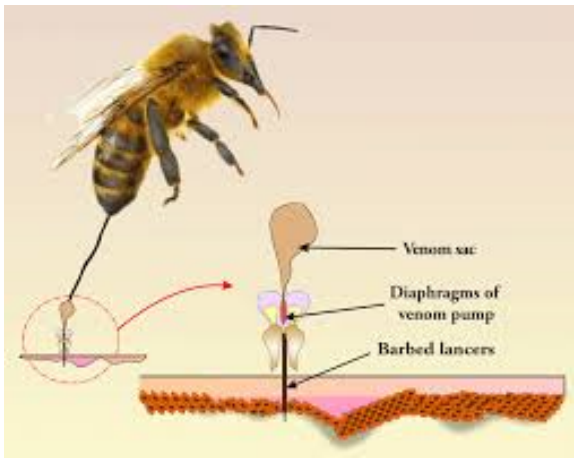


A **bee sting** is a wound caused by the stinger from a bee (honey bee, bumblebee, sweat bee, etc.) being injected into one's flesh. The stings of most of these species can be quite painful, and are therefore keenly avoided by many people.

Bee stings differ from insect bites, and the venom or toxin of stinging insects is quite different. Therefore, the body's reaction to a bee sting may differ significantly from one species to another. In particular, bee stings are acidic, whereas wasp stings are alkaline, so the body's reaction to a bee sting may be very different from its reaction to a wasp sting.

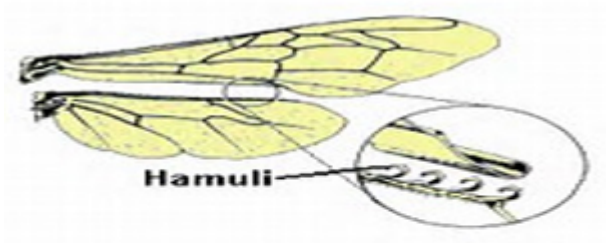
The most aggressive stinging insects are vespid wasps (including bald-faced hornets and other yellowjackets) and hornets (especially the Asian giant hornet). All of these insects aggressively defend their nests.

Although for most people a bee sting is painful but otherwise relatively harmless, in people with insect sting allergy, stings may trigger a dangerous anaphylactic reaction that is potentially deadly. Additionally, honey bee stings release pheromones that prompt other nearby bees to attack.

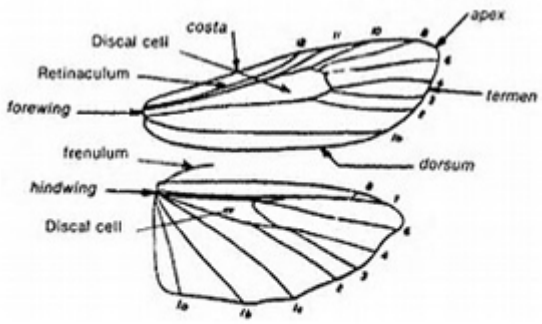


Coupling device of Honey bee

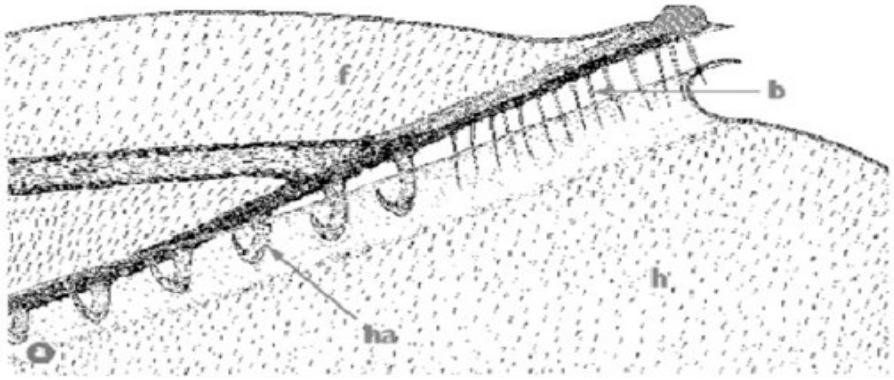
These structures are located near the proximal end of the anterior margin of each hindwing and which couple it to a fold in the forewing to form a single, functional aerofoil during flight.



Hamuli in Hymenoptera |



Frenulum in lepidoptera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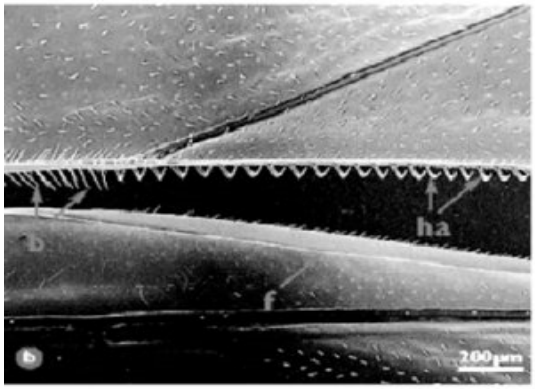
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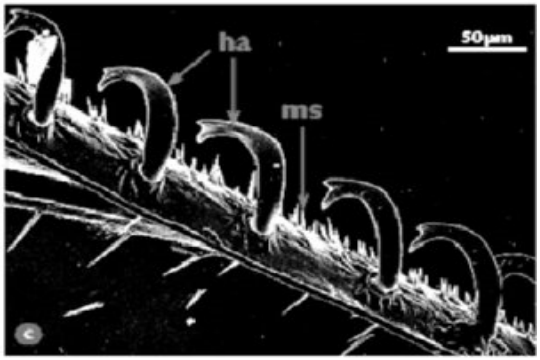
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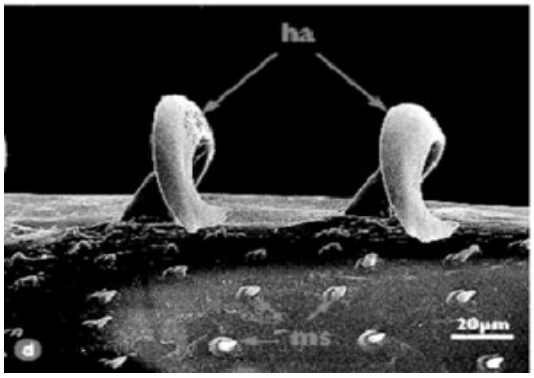
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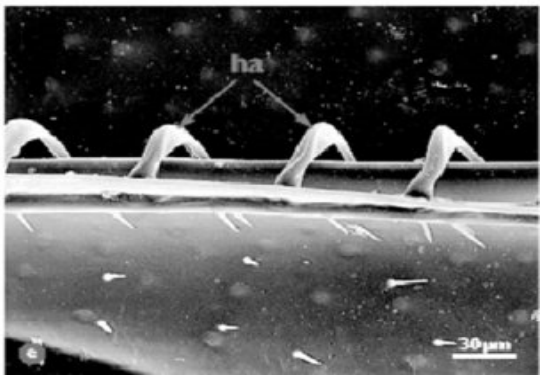
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Dose Response Curve

Dose-Response Curve:

The dose–response relationship, or exposure–response relationship, describes the magnitude of the response of an organism, as a function of exposure (or doses) to a stimulus or stressor (usually a chemical) after a certain exposure time. Dose–response relationships can be described by dose–response curves. This is explained further in the following sections. A stimulus response function or stimulus response curve is defined more broadly as the response from any type of stimulus, not limited to chemicals.

Importance:

Studying dose response, and developing dose–response models, is central to determining "safe", "hazardous" and (where relevant) beneficial levels and dosages for drugs, pollutants, foods, and other substances to which humans or other organisms are exposed. Dose response relationships may be used in individuals or in populations.

In populations, dose–response relationships can describe the way groups of people or organisms are affected at different levels of exposure.

Dose response relationships modelled by dose response curves are used extensively in pharmacology and drug development.

In particular, the shape of a drug's dose–response curve (quantified by EC50, nH and ymax parameters) reflects the biological activity and strength of the drug.

Construction of dose-response curves:

A dose–response curve is a coordinate graph relating the magnitude of a stimulus to the response of the receptor. A number of effects (or endpoints) can be studied. The measured dose is generally plotted on the X axis and the response is plotted on the Y axis. In some cases, it is the logarithm of the dose

that is plotted on the X axis, and in such cases the curve is typically sigmoidal, with the steepest portion in the middle. Biologically based models using dose are preferred over the use of log(dose) because the latter can visually imply a threshold dose when in fact there is none.

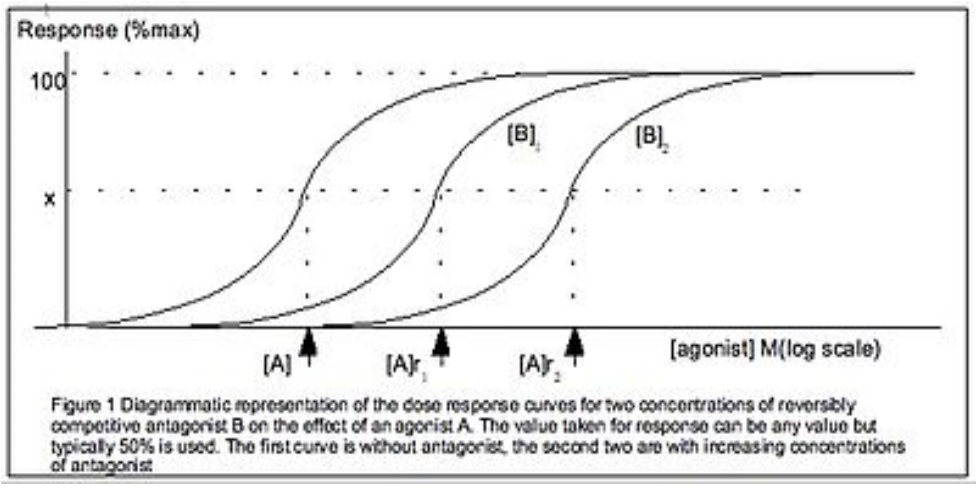


Figure 1 Diagrammatic representation of the dose response curves for two concentrations of reversibly competitive antagonist B on the effect of an agonist A. The value taken for response can be any value but typically 50% is used. The first curve is without antagonist, the second two are with increasing concentrations of antagonist

Fig: Semi-log plots of the hypothetical response to agonist, log concentration on the x-axis, in combination with different antagonist concentrations. The parameters of the curves, and how the antagonist changes them, gives useful information about the agonist's pharmacological profile. This curve is similar but distinct from that, which is generated with the ligand-bound receptor concentration on the y-axis.

Statistical analysis of dose–response curves may be performed by regression methods such as the probit model or logit model, or other methods such as the Spearman-Karber method.

Hill Equation:

The Hill equation is the following formula, where [E]is the magnitude of the response. [A] is the drug concentration (or equivalently, stimulus intensity) and EC50 is the drug concentration that produces a 50% maximal response and ‘n’ in the Hill coefficient.

$$E/E_{max}= 1/1+(EC_{50}/[A])^{n}$$

The parameters of the dose response curve reflect measures of potency (such as EC50, IC50, ED50, etc.) and measures of efficacy (such as tissue, cell or population response).

A commonly used dose–response curve is the EC50 curve, the half maximal effective concentration, where the EC50 point is defined as the inflection point of the curve.

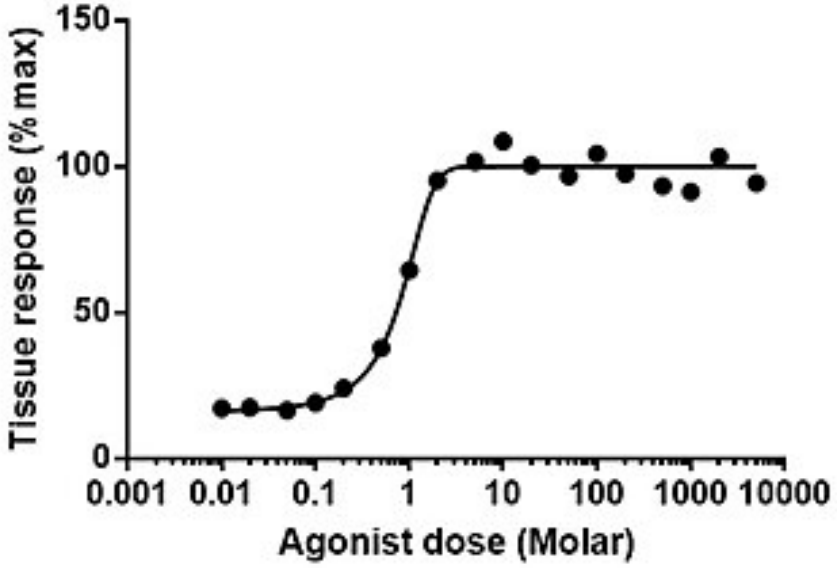


Fig:A dose response curve showing the normalised tissue response to stimulation by an agonist. Low doses are insufficient to generate a response, while high doses generate a maximal response. The steepest point of the curve corresponds with an EC50 of 0.7 molar

Advantages:

The dose-response curves have a number of utility values such as, -

i. Helps in determiningtoxicity of substances.

ii. Identification of threshold limit of toxicity.

iii. Helps in determining LD50, LC50, ED50 etc.

Disadvantages:

The concept of linear dose–response relationship, thresholds, and all-or-nothing responses may not apply to non-linear situations. A threshold model or linear no-threshold model may be more appropriate, depending on the circumstances.

Calculation of LC50 Value Using Experimental Data

Introduction:

LC50 is short for "lethal concentration 50%" or "median lethal concentration". It is the concentration of a substance (in air or water) at which half the members of a population are killed after a specified duration of exposure. Exposure is typically through inhalation. This value is very important in toxicology and agriculture as it gives an indication of substance (eg. herbicide) toxicity. The LC50 is inversely proportional to toxicity. A substance with a lower LC50 is more toxic than one which has a higher LC50.

Experiment:

Traditionally, the concentrations (x-value) are entered as mass of substance per volume of either air or solution. The units commonly used are milligram per liter (mg/L) or parts per million (ppm). The responses (y-value) are typically entered as percentage of population killed (range from 0 to 100). This will yield an upward-slopping sigmoidal curve. If percentage survival is used instead, a downward-slopping sigmoid al curve will be generated.

In this experiment, lethal concentration (LC50) values of Gamaxin on minor carp (Puntius sarana) were investigated. In practice, experimental setup

was constituted 100 fish (a total of 110 fish with 10 control fish) to be placed in ten replicates. Gamaxin was added into aquariums at the doses of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ppm and determined mortality times of carp exposed to these concentrations. Percentage death of fish calculated in these concentrations.

24 hr test is the first step and observe the group of fish exposed. So count the number of dead and live organism, including the control group at the 24 hours after starting the test. Remove dead organisms as soon as they are observed. Use Abbot's Formula, to find the corrected percentage mortality of organism in each group.

Experimental Result:

Sl. No.	Conc^{n} of Toxicant in ppm	No. of Test Organisms	After 24 Hrs.		After 48 Hrs.	
			Alive	Dead	Alive	Dead
1	0.1	10	10	0	9	1
2	0.2	10	10	0	8	2
3	0.3	10	9	1	7	3
4	0.4	10	8	2	5	5
5	0.5	10	7	3	4	6
6	0.6	10	6	4	3	7
7	0.7	10	5	5	2	8
8	0.8	10	3	7	2	8
9	0.9	10	2	8	0	10
10	1.0	10	1	9	0	10

Comment:

The graph based on experimental result clearly shows that there are two different LC50 values for two different exposure times. The percentage of mortality is directly proportional to the concentration of the toxicant as well as to the exposure time. So not only the toxicant concentration but also the

exposure time has a great impact on mortality. Lower LC50 value indicates the higher toxicity and higher LC50 value indicates lower toxicity. The LC50 values for 24 hrs. and 48 hrs. of exposure time are 0.7 ppm and 0.4 ppm respectively.

MATC

Introduction:

The maximum acceptable toxicant concentration (MATC) is a value that is calculated through aquatic toxicity tests to help set water quality regulations for the protection of aquatic life. Using the results of a partial life-cycle chronic toxicity test, the MATC is reported as the geometric mean between the No Observed Effect Concentration (NOEC) and the lowest observed effect concentration (LOEC).

The MATC is used to set regulatory standards for priority pollutants under the US federal Clean Water Act. Regulatory guidelines give two acceptable concentrations of pollutants to protect against effects: chronic or acute. Since the MATC should only be reported in chronic toxicity tests, there is a widely accepted method to convert the chronic MATC to a concentration that protects against acute effects.

The MATC is calculated and reported from the results of a number of standard procedures designed by the United States Environmental Protection Agency (US EPA) and other organizations to maintain high accuracy and precision among all toxicity tests for regulatory purposes.

Calculations:

Chronic toxicity test:

The NOEC and LOEC are derived as a comparison from the negative control, or the experimental group that does not contain the chemical in question. The NOEC is the highest concentration that does not cause a statistically different effect than the negative control through statistical hypothesis testing. Likewise, the LOEC is the lowest concentration tested that does cause a statistically different effect than the negative control. The MATC is the geometric mean between these two values, such that: $MATC=\sqrt{(NOEC)(LOEC)}$

The MATC is calculated to protect against chronic effects on overall function or health of an organism, not death. A partial life cycle test must be used. This type of toxicity test uses organisms in their most sensitive life stages, usually during times of early reproduction and growth, but not juveniles. The MATC is the highest concentration that should not cause chronic effects.

Applying MATC to acutely toxic concentrations:

The MATC can be applied to the results of an acute toxicity test to obtain a concentration that would protect against adverse effects during an acute exposure. An LC50, or the concentration at which 50% of the organisms die during an acute toxicity test is used to derive a value called the acute to chronic ratio (ACR).

The MATC can be used to calculate the ACR as follows: $ACR=LC50/MATC$

The ACR is useful for estimating an MATC for species in which only acute toxicity data exists.

Advantages:

NOEC and LOEC's were used more often in the past, and there are more test results reporting NOEC and LOEC's than EC10's. The time and effort required to perform all of the previous tests to derive a different value is not seen as a good use of resources. In addition, the use of NOEC and LOEC's allows for reporting of one number to regulatory agencies.

Disadvantages:

There are no confidence intervals to show a measure of uncertainty in a NOEC and LOEC. In addition, the NOEC and LOEC can only be concentrations in the test, and nothing in between. Because of these reasons, values that are derived through curve fitting methods, such as an LC50, or EC10 (the concentration that causes the measured effect in 10% of organisms) would be preferred if it was possible more often.

Microbiology

DIFFERENTIAL STAINING TECHNIQUES

Viewing Bacterial Cells

The microscope is a very important tool in microbiology, but there are limitations when it comes to using one to observe cells in general and bacterial cells in particular. Two of the most important concerns are resolution and contrast. Resolution is a limitation that we can't do much about, since most bacterial cells are already near the resolution limit of most light microscopes. Contrast, however, can be improved by either using a different type of optical system, such as phase contrast or a differential interference contrast microscope, or by staining the cells (or the background) with a chromogenic dye that not only adds contrast, but gives them a color as well.

There are many different stains and staining procedures used in microbiology. Some involve a single stain and just a few steps, while others use multiple stains and a more complicated procedure. Before you can begin the staining procedure, the cells have to be mounted (smeared) and fixed onto a glass slide.

A bacterial smear is simply that—a small amount of culture spread in a very thin film on the surface of the slide. To prevent the bacteria from washing away during the staining steps, the smear may be chemically or physically "fixed" to the surface of the slide. Heat fixing is an easy and efficient method, and is accomplished by passing the slide briefly through the flame of a Bunsen burner, which causes the biological material to become more or less permanently affixed to the glass surface.

Heat fixed smears are ready for staining. In a simple stain, dyes that are either attracted by charge (a cationic dye such as methylene blue or crystal violet) or repelled by charge (an anionic dye such as eosin or India ink) are added to the smear. Cationic dyes bind the bacterial cells which can be easily observed against the bright background. Anionic dyes are repelled by the cells, and therefore the cells are bright against the stained background. See Figures 1 and 2 for examples of both.

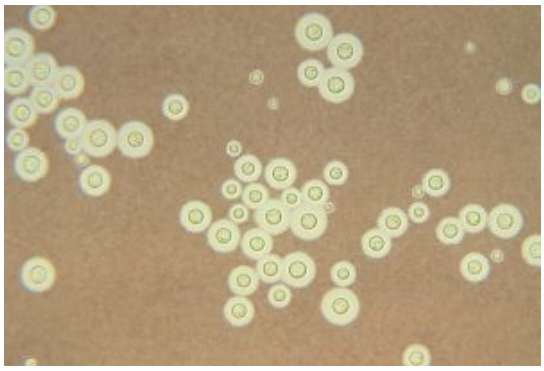


Figure 1. Negative stain of *Cyptococcus neoformans*, an encapsulated yeast

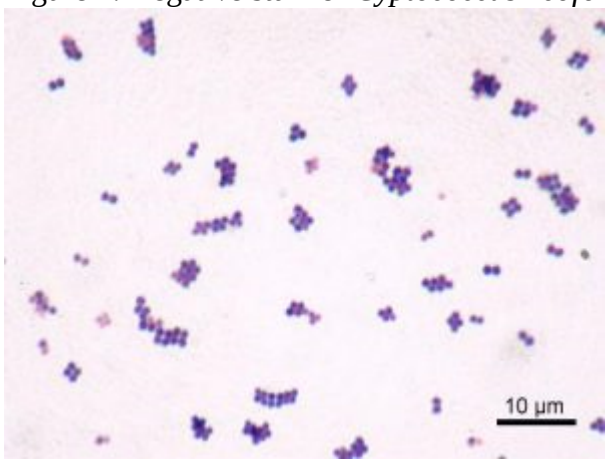


Figure 2. Positive stain of *Staphylococcus aureus.*

Probably the most important feature made obvious when you stain bacterial cells is their **cellular morphology** (not to be confused with colonial morphology, which is the appearance of bacterial colonies on an agar plate). Most heterotrophic and culturable bacteria come in a few basic shapes: spherical cells (coccus/cocci), rod-shaped cells (bacillus/bacilli), or rod-shaped cells with bends or twists (vibrios and spirilla, respectively). There is greater diversity of shapes among Archaea and other bacteria found in ecosystems other than the human body.

Often bacteria create specific **arrangements** of cells, which form as a result of binary fission by the bacteria as they reproduce. Arrangements are particularly obvious with non-motile bacteria, because the cells tend to stay together after the fission process is complete. Both the shape and arrangement of cells are characteristics that can be used to distinguish among bacteria. The most commonly encountered bacterial shapes (cocci and bacilli) and their possible arrangements are shown in Figures 3 and 4.

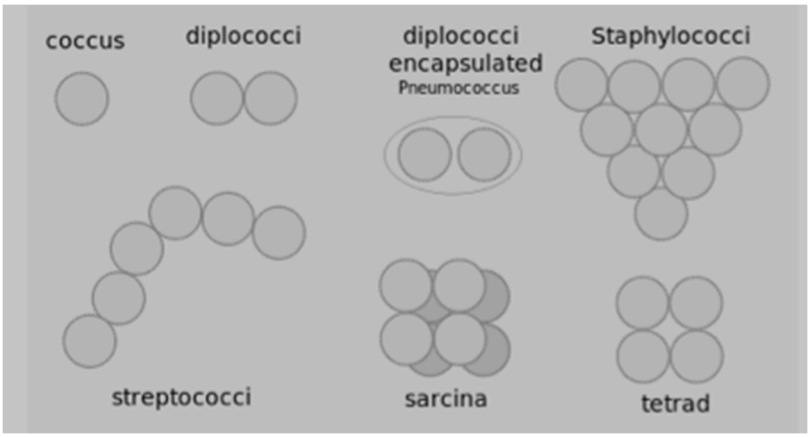


Figure 3. Possible bacterial cell arrangements for cocci

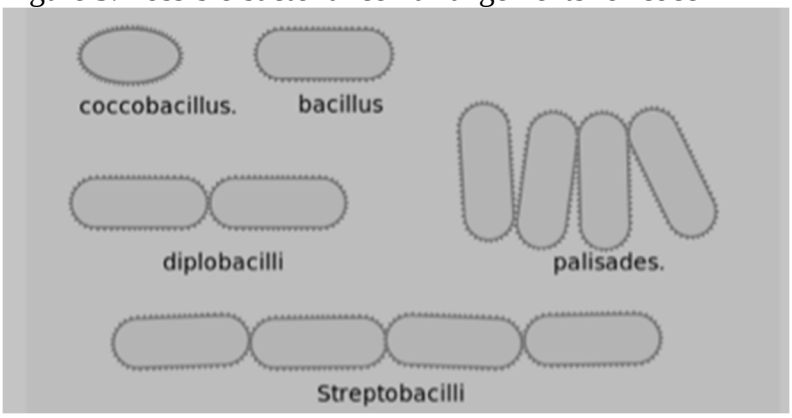


Figure 4. Possible bacteria cell arrangements for bacilli

Differential Staining Techniques

In microbiology, differential staining techniques are used more often than simple stains as a means of gathering information about bacteria. Differential staining methods, which typically require more than one stain and several steps, are referred to as such because they permit the differentiation of cell types or cell structures. The most important of these is the Gram stain. Other differential staining methods include the endospore stain (to identify endospore-forming bacteria), the acid-fast stain (to discriminate *Mycobacterium* species from other bacteria), a metachromatic stain to identify phosphate storage granules, and the capsule stain (to identify encapsulated bacteria). We will be performing the Gram stain and endospore staining procedures in lab, and view prepared slides that highlight some of the other cellular structures present in some bacteria.

Gram Stain

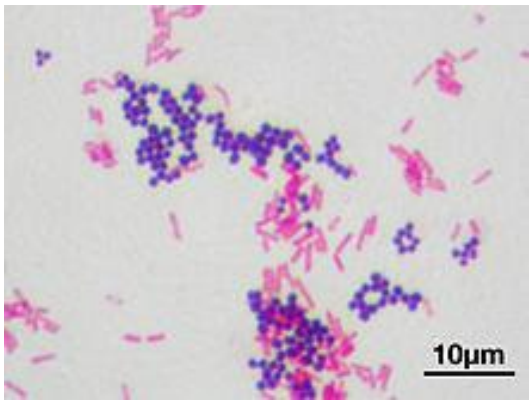


Figure 5. Bacteria stained with Gram stain.

In 1884, physician Hans Christian Gram was studying the etiology (cause) of respiratory diseases such as pneumonia. He developed a staining procedure that allowed him to identify a bacterium in lung tissue taken from deceased patients as the etiologic agent of a fatal type of pneumonia. Although it did little in the way of treatment for the disease, the Gram stain method made it much easier to diagnose the cause of a person's death at autopsy. Today we use Gram's staining techniques to aid in the identification of bacteria, beginning with a preliminary classification into one of two groups:

Gram positive or **Gram negative**.

The differential nature of the Gram stain is based on the ability of some bacterial cells to retain a primary stain (crystal violet) by resisting a decolorization process. Gram staining involves four steps. First cells are stained with crystal violet, followed by the addition of a setting agent for the stain (iodine). Then alcohol is applied, which selectively removes the stain from only the Gram negative cells. Finally, a secondary stain, safranin, is added, which counterstains the decolorized cells pink.

Although Gram didn't know it at the time, the main difference between these two types of bacterial cells is their cell walls. Gram negative cell walls have an outer membrane (also called the envelope) that dissolves during the alcohol wash. This permits the crystal violet dye to escape. Only the decolorized cells take up the pink dye safranin, which explains the difference in color between the two types of cells. At the conclusion of the Gram stain procedure, Gram positive cells appear purple, and Gram negative cells appear pink.

When you interpret a Gram stained smear, you should also describe the morphology (shape) of the cells, and their arrangement. In Figure 5, there are two distinct types of bacteria, distinguishable by Gram stain reaction, and also by their shape and arrangement. Below, describe these characteristics for both bacteria:

	Gram positive bacterium:	**Gram negative bacterium:**
Morphology		
Arrangement		

Acid Fast Stain

Some bacteria produce the waxy substance **mycolic acid** when they construct their cell walls. Mycolic acid acts as a barrier, protecting the cells from dehydrating, as well as from phagocytosis by immune system cells in a host. This waxy barrier also prevents stains from penetrating the cell, which is why the Gram stain does not work with mycobacteria such as *Mycobacterium*, which are pathogens of humans and animals. For these bacteria, the **acid–fast staining** technique is used.

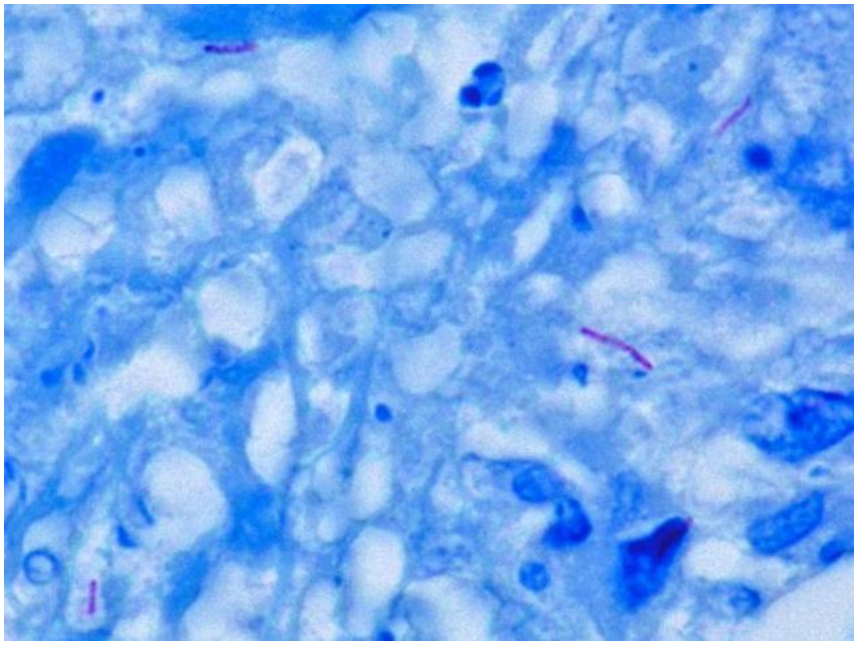


Figure 6. Acid-fast bacilli in sputum

To perform the acid-fast stain, a heat-fixed smear is flooded with the primary stain carbol fuchsin, while the slide is heated over a steaming water bath. The heat “melts” the waxy cell wall and permits the absorption of the dye by the cells. Then the slide is allowed to cool and a

solution of acid and alcohol is added as a decolorizer. Cells that are “acid-fast” because of the mycolic acid in their cell wall resist decolorization and retain the primary stain. All other cell types will be decolorized. Methylene blue is then used as a counterstain. In the end, acid-fast bacteria (AFB) will be stained a bright pink color, and all other cell types will appear blue.

Staining Methods to Highlight Specific Cell Structures

Capsule: The polysaccharide goo that surrounds some species of bacteria and a few types of eukaryotic microbes is best visualized when the cells are negative stained. In this method, the bacteria are first mixed with the stain, and then a drop of the mixture is spread across the surface of a slide in the thin film. With this method, capsules appear as a clear layer around the bacterial cells, with the background stained dark.

Metachromatic granules or other intracytoplasmic bodies: Some bacteria may contain storage bodies that can be stained. One example is the Gram positive bacilli *Corynebacterium*, which stores phosphate in structures called “volutin” or metachromatic granules that are housed within the cell membrane. Various staining methods are used to visualize intracytoplasmic bodies in bacteria, which often provide an identification clue when observed in cells.

Endospore Stain

Endospores are dormant forms of living bacteria and should not be confused with reproductive spores produced by fungi. These structures are produced by a few genera of Gram-positive bacteria, almost all bacilli, in response to adverse environmental conditions. Two common bacteria that produce endospores are *Bacillus* or *Clostridum*. Both live primarily in soil and as symbionts of plants and animals, and produce endospores to survive in an environment that change rapidly and often.

The process of **endosporulation** (the formation of endospores) involves several stages. After the bacterial cell replicates its DNA, layers of peptidoglycan and protein are produced to surround the genetic material. Once fully formed, the endospore is released from the cell and may sit dormant for days, weeks, or years. When more favorable environmental conditions prevail, endospores **germinate** and return to active duty as vegetative cells.

Mature endospores are highly resistant to environmental conditions such as heat and chemicals and this permits survival of the bacterial species for very long periods. Endospores formed millions of years ago have been successfully brought back to life, simply by providing them with water and food.

Because the endospore coat is highly resistant to staining, a special method was developed to make them easier to see with a brightfield microscope. This method, called the **endospore stain**, uses either heat or long exposure time to entice the endospores to take up the primary stain, usually a water soluble dye such as malachite green since endospores are permeable to water. Following a decolorization step which removes the dye from the vegetative cells in the smear, the counterstain safranin is applied to provide color and contrast. When stained by this method, the endospores are green, and the vegetative cells stain pink, as shown in Figure 7.

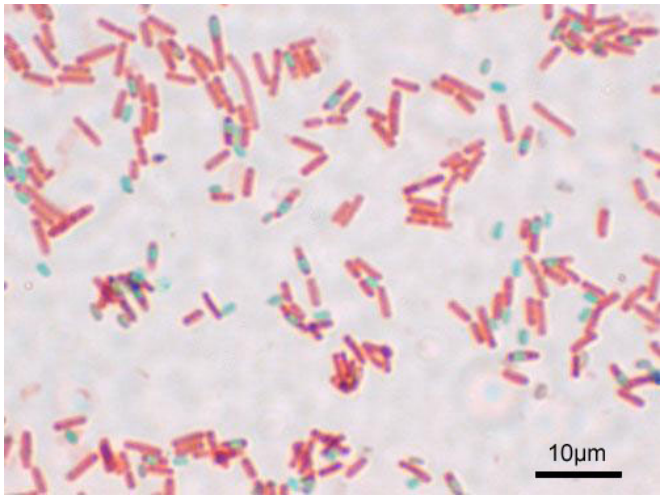


Figure 7. Bacterial cells with endospores, stained with the endospore stain.

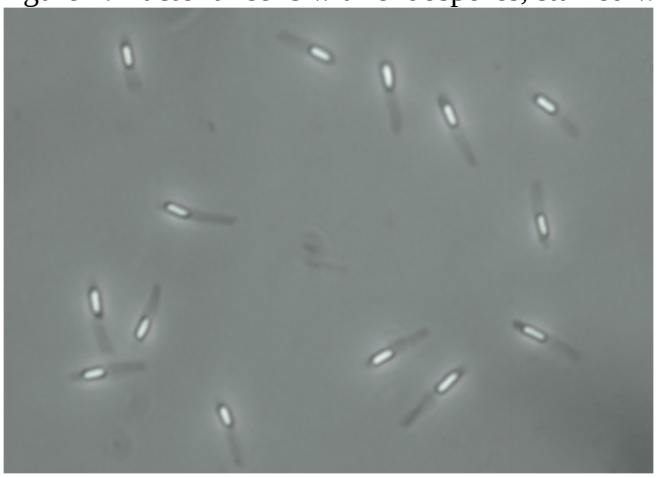


Figure 8. Bacilli with endospores viewed by phase-contrast microscopy.

Although endospores themselves are resistant to the Gram stain technique, bacterial cells captured in the process of creating these structures can be stained. In this case, the endospores are seen as clear oval or spherical areas within the stained cell. Endospores can also be directly observed in cells by using phase contrast microscopy, as shown in Figure 8.

Method

Because many differential staining methods require several steps and take a long time to complete, we will not be performing all of the differential staining methods discussed above.

Pre-stained slides will be used to visualize bacterial capsules, metachromatic granules, and acid-fast bacilli. Obtain one slide of each of the three bacteria listed in the table below. As you view these slides, make note of the "highlighted" structures. Your environmental isolate may have one or more of these cellular features, and learning to recognize them will aid in identification. These should all be viewed using the oil immersion objective lens.

Bacterium	Stain	Description or sketch of cells with the specified feature
Flavobacterium capsulatum	Capsule stain	
Corynebacterium diphtheriae	Methylene blue(metachromatic granules)	
Mycobacterium tuberculosis	Acid fast stain	

Gram Stain

All staining procedures should be done over a sink. The Gram stain procedure will be demonstrated, and an overview is provided in Table 1.

Table 1. Gram stain procedural steps.

Step	Procedure	Outcome
Primary stain(crystal violet)	Add several drops of crystal violet to the smear and allow it to sit for 1 minute. Rinse the slide with water.	Both Gram-positive and Gram-negative cells will be stained purple by the crystal violet dye.
Mordant (iodine)	Add several drops of iodine to	Iodine "sets" the crystal violet, so

	the smear and allow it to sit for 1 minute. Rinse the slide with water.	both types of bacteria will remain purple.
Decolorization (ethanol)	Add drops of ethanol one at a time until the runoff is clear. Rinse the slide with water.	Gram-positive cells resist decolorization and remain purple. The dye is released from Gram-negative cells.
Counterstain(safranin)	Add several drops of safranin to the smear and allow it to sit for one minute. Rinse the slide with water and blot dry.	Gram-negative cells will be stained pink by the safranin. This dye has no effect on Gram-positive cells, which remain purple.

A volunteer from your lab bench should obtain cultures of the bacteria you will be using in this lab, as directed by your instructor. One of the cultures will be a Gram positive bacterium, and the other will be Gram negative. Below, write the names of the bacteria you will be using, along with the BSL for each culture:

Obtain two glass slides, and prepare a smear of each of the two bacterial cultures, one per slide, as demonstrated. Allow to COMPLETELY air dry and heat fix. Stain both smears using the Gram stain method. Observe the slides with a light microscope at 1,000X and record your observations in the table below.

Name of culture Gram stain reaction Cellular morphology Arrangement

Gram Stain “Final Exam”: prepare a smear that contains a mixture of the Gram-positive AND Gram-negative bacteria by adding a small amount of each bacterium to a single drop of water on a slide. Heat fix the smear and Gram stain it. You should be able to determine the Gram stain reaction, cellular morphology and arrangement of BOTH bacteria in this mixed smear. Your instructor may ask to see this slide and offer constructive commentary.

Endospore Stain

Only a few genera of bacteria produce endospores and nearly all of them are Gram-positive bacilli. Most notable are *Bacillus* and *Clostridium* species, which naturally live in soil and are common contaminants on surfaces. The growth of *Clostridium* spp. is typically limited to anaerobic environments; *Bacillus* spp. may grow aerobically and anaerobically. Endospore-forming bacteria are distinct from other groups of Gram positive bacilli and distinguishable by their endospores.

An overview of the endospore stain procedure is provided in Table 2.

Table 2. Endospore stain procedural steps.

Step	Procedure	Outcome
Primary stain(malachite green)	Add several drops of malachite green to the smear and allow it to sit for 10 minutes. If the stain starts to dry out, add additional drops.	Vegetative cells will immediately take up the primary stain. Endospores are resistant to staining but eventually take up the dye.
Decolorization(water)	Rinse the slide under a gentle stream of water for 10-15 seconds.	Once the endospores are stained, they remain green. A thorough rinse with water will decolorize the vegetative cells.
Counterstain(safranin)	Add several drops of safranin to the smear and allow it to sit for 1 minute. Rinse the slide and blot dry.	Decolorized vegetative cells take up the counterstain and appear pink; endospores are light green.

After staining, endospores typically appear as light green oval or spherical structures, which may be seen either within or outside of the vegetative cells, which appear pink.

The shape and location of the endospores inside the bacterial cells, along with whether the sporangium is either distending (D) or not distending (ND) the sides of the cell, are important characteristics that aid in differentiating among species (see Figure 9).

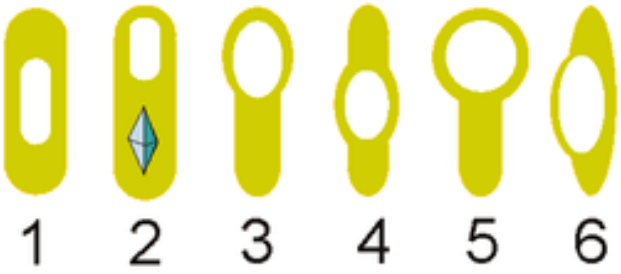


Figure 9

1. Oval, central, not distended (ND)
2. Oval, terminal, ND (and parasporal crystal)
3. Oval, terminal, distended (D)
4. Oval, central, D
5. Spherical, terminal, D
6. Oval, lateral, D

Endospores are quite resistant to most staining procedures; however, in a routinely stained smear, they may be visible as “outlines” with clear space within. If you observe “outlines” or what appear to be “ghosts” of cells in a Gram stained smear of a Gram-positive bacilli, then the endospore stain should also be performed to confirm the presence or absence of endospores.

GRAM STAINING

Introduction:

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decoloring process.

Principle

Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolorization, and counterstaining, usually with safranin. Due to

differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria, Gram positive bacteria (with a thicker peptidoglycan layer) retain crystal violet stain during the decolorization process, while Gram negative bacteria lose the crystal violet stain and are instead stained by the safranin in the final staining process. The process involves three steps:

1. Cells are stained with crystal violet dye. Next, a Gram's iodine solution (iodine and potassium iodide) is added to form a complex between the crystal violet and iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water.

2. A decolorizer such as ethyl alcohol or acetone is added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. The large crystal violet-iodine complex is not able to penetrate this tightened peptidoglycan layer, and is thus trapped in the cell in Gram positive bacteria. Conversely, the the outer membrane of Gram negative bacteria is degraded and the thinner peptidoglycan layer of Gram negative cells is unable to retain the crystal violet-iodine complex and the color is lost.

3. A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram positive cells. However, the decolorized Gram negative cells are stained red.

Reagents

- Crystal violet (primary stain)[100%]
- Iodine solution/Gram's Iodine (mordant that fixes crystal violet to cell wall)
- Decolorizer (e.g. ethanol)
- Safranin (secondary stain) [0.5–1% solution in water or 50% ethanol]
- Water (preferably in a squirt bottle)

Procedure

1. Make a slide of cell sample to be stained. Heat fix the sample to the slide by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner three times.
2. Add the primary stain (crystal violet) to the sample/slide and incubate for 1 minute. Rinse slide with a gentle stream of water for a maximum of 5 seconds to remove unbound crystal violet.
3. Add Gram's iodine for 1 minute- this is a mordant, or an agent that fixes the crystal violet to the bacterial cell wall.
4. Rinse sample/slide with acetone or alcohol for ~3 seconds and rinse with a gentle stream of water. The alcohol will decolorize the sample if it is Gram negative, removing the crystal violet. However, if the alcohol remains on the sample for too long, it may also decolorize Gram positive cells.
5. Add the secondary stain, safranin, to the slide and incubate for 1 minute. Wash with a gentle stream of water for a maximum of 5 seconds. If the bacteria is Gram positive, it will retain the primary stain (crystal violet) and not take the secondary stain (safranin), causing it to look violet/purple under a microscope. If the bacteria is Gram

negative, it will lose the primary stain and take the secondary stain, causing it to appear red when viewed under a microscope.

Observation: Gram-positive bacteria appear blue or violet and gram-negative bacteria appear pinkish red.

Methods of Sterilization

Sterilization is necessary for the complete destruction or removal of all microorganisms (including spore-forming and non-sporeforming bacteria, viruses, fungi, and protozoa) that could contaminate pharmaceuticals or other materials and thereby constitute a health hazard. Since the achievement of the absolute state of sterility cannot be demonstrated, the sterility of a pharmaceutical preparation can be defined only in terms of probability.

Classical sterilization techniques using saturated steam under pressure or hot air are the most reliable and should be used whenever possible. Other sterilization methods include filtration, ionizing radiation (gamma and electron-beam radiation), and gas (ethylene oxide, formaldehyde).

Heating in an autoclave (steam sterilization) Exposure of microorganisms to saturated steam under pressure in an autoclave achieves their destruction by the irreversible denaturation of enzymes and structural proteins. The temperature at which denaturation occurs varies inversely with the amount of water present. Sterilization in saturated steam thus requires precise control of time, temperature, and pressure. As displacement of the air by steam is unlikely to be readily achieved, the air should be evacuated from the autoclave before admission of steam.

The recommendations for sterilization in an autoclave are 15 minutes at 121-124 °C (200 kPa).The temperature should be used to control and monitor the process; the pressure is mainly used to obtain the required steam temperature. Minimum sterilization time should be measured from the moment when all the materials to be sterilized have reached the required temperature throughout.

Aqueous solutions in glass containers usually reach thermal equilibrium within 10 minutes for volumes up to 100 mL and 20 minutes for volumes up to 1000 mL.

Porous loads, such as surgical dressings and related products, should be processed in an apparatus that ensures steam penetration. Most dressings are adequately sterilized by maintaining them at a temperature of 134 - 138 °C for 5 minutes. In certain cases, glass, porcelain, or metal articles are sterilized at 121 - 124 °C for 20 minutes.

Fats and oils may be sterilized at 121 °C for 2 hours but, whenever possible, should be sterilized by dry heat.

Dry-heat sterilization In dry-heat processes, the primary lethal process is considered to be oxidation of cell constituents. Dry-heat sterilization requires a higher temperature than moist heat and a longer exposure time. The method is, therefore, more convenient for heat-stable, non-aqueous materials that cannot be sterilized by steam because of its deleterious effects or failure to penetrate. Such materials include glassware, powders, oils, and some oil-based injectables.

Filtration Sterilization by filtration is employed mainly for thermolabile solutions. These may be sterilized by passage through sterile bacteria-retaining filters, e.g. membrane filters (cellulose derivatives, etc.), plastic, porous ceramic, or suitable sintered glass filters, or combinations of these. Asbestos-containing filters should not be used.

Radiation UV, x-rays and gamma rays are all types of electromagnetic radiation that have profoundly damaging effects on DNA, so make excellent tools for sterilization.The main difference between them, in terms of their effectiveness, is their penetration. UV has limited penetration in air so sterilisation only occurs in a fairly small area around the lamp. However, it is relatively safe and is quite useful for sterilising small areas, like laminar flow hoods. X-rays and gamma rays are far more penetrating, which makes them more dangerous but very effective for large scale cold sterilization of plastic items (e.g. syringes) during manufacturing.

PREPARATION OF DIFFERENT CULTURE MEDIA

The culture media (nutrients) consist of chemicals which support the growth of culture or microorganisms. Microbes can use the nutrients of culture media as their food is necessary for cultivating them in vitro.

On the basis of chemical composition, the culture media are classified into two types:

(i) Synthetic or chemically defined medium:

These media are prepared by mixing all the pure chemicals of known composition for e.g. Czapek Dox medium.

(ii) Semi-synthetic or undefined medium:

Such are those media, where exact chemical composition is unknown e.g. potato dextrose agar or MacConkey agar medium.

On the basis of consistency, the culture media are of three types:

(a) Solid medium or synthetic medium:

When 5-7% agar agar or 10-20% gelatin is added the liquid broth becomes solidified. Such media are used for making agar slants or slopes and agar stab.

(b) Liquid medium or broth:

In such cases no agar is added or used while preparing the medium. After inoculation and later incubation, the growth of cells becomes visible in the form of small mass on the top of the broth.

(c) Semi-solid or floppy agar medium:

Such media are prepared by adding half quantity of agar (1/2 than required for solid medium) i.e. about 0.5% in the medium. This type of medium may be selective which promote the growth of one organism and retards the growth of the other organism. On the other hand, there are differential media which serve to differentiate organisms growing together.

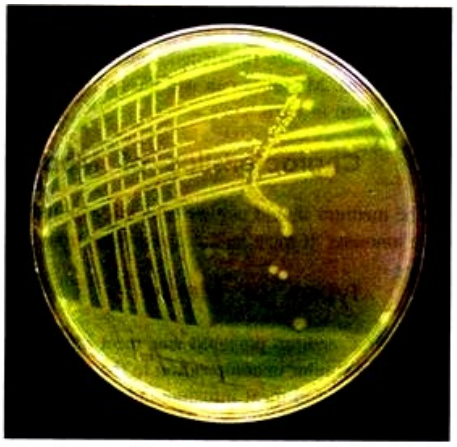
Preparation of Medium:

The liquid medium or broth is prepared by dissolving the known amounts of chemicals in distilled water; the pH is adjusted by adding N/10 HCl or 1N NaOH. The liquid medium is dissolved into either Erlenmeyer flasks or rimless clean test tubes.

In 15 ml capacity of test tube, 5 ml medium should be poured while in flask of 250 ml capacity, the amount of the medium should be 100 ml. These are then plugged with non-adsorbent cotton plugs. The plugged tubes or flasks should be wrapped by brown paper and placed for sterilization by autoclaving at a pressure of 15 lbs/inch^2 (at temperature 121°C), for 15 min.

The heat sensitive substances (protein or enzymes etc.) should be sterilized by using membrane filters (millipore). The agar agar is to be dissolved separately and dispensed after dissolving all ingredients of the medium. It is first to be noted that all the glassware in use should be sterilized in oven at 170°C for 3 h before using them. Such sterilized glassware is needed for pouring the medium used for culturing the microorganisms.

Each and every biological process requires energy for their vital activities. The basic cell building requirements are supplied by the nutrition, which is manipulated according to its requirement. Nutrition not only provides energy but also acts as precursors for growth of microorganisms. The nutritional requirement of an organism depends upon the biochemical capacity. If an organism is capable of synthesizing its own food using various inorganic components, requires a simple nutritional diet whereas organism unable to meet such synthesis requires complex organic substances.



A culture medium prepared for microbial testing

INOCULATION OF MICROBES TO RESPECTIVE CULTURE MEDIA THROUGH PROPER CULTURE METHODS

There are different methods of inoculation used in bacteriology. However, the most common methods are described below:

Streak plating technique

The purpose of this procedure (outlined below) is to obtain well isolated colonies from a specimen or culture inoculum by creating areas of increasing dilution on a single plate13:

1. Inoculate clinical specimen using a sterile inoculation loop onto agar media. Spread specimen over a portion of the culture media surface gently (see figure 1)

2. Drag loop from the inoculated section and spread it out into a second section

3. Drag loop from the section 2 and then spread it out into the third section. Do the same for the third and the fourth section. Ensure that sections 1 and 4 do not overlap. Dispose of the inoculation loop used into an appropriate container (see figures 2 and 3)

4. Replace the lid and then incubate the streaked agar plate at the appropriate temperature in an inverted position to prevent condensation

Agar stab technique

The method (outlined below) is used to prepare stab cultures (to observe motility, or when inoculating certain types of solid medium) and to pick single colonies from a plate:

1. Using aseptic technique pick a single well-isolated colony with a sterile inoculating stab needle and stab the needle several times through the agar to the bottom of the vial or tube

2. Replace the cap and tighten loosely when incubating to allow gas exchange

3. Incubate the stabbed agar plate/slant at the appropriate temperature

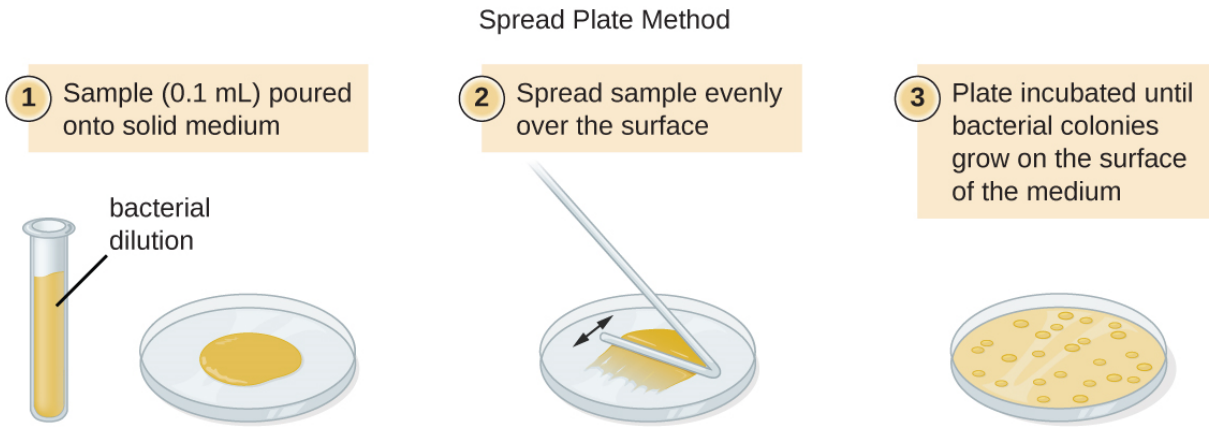
Spread plate technique

The purpose of this procedure (outlined below) is to distribute cells evenly so that well isolated individual colonies can be grown. These are then counted or used for further tests such as serial dilutions:

1. Inoculate clinical specimen using a sterile spreader or alternative onto agar media. Gently spread bacteria over the entire culture medium surface backward and forward while rotating the plate. Avoid the spreader touching the edges of the agar plate

2. Replace the lid and allow the plate to stand in an upright position (with the lid at the top) to dry for 10 to 20 minutes14

3. Incubate the spread agar plate at the appropriate temperature in an inverted position (with the lid at the bottom)



ENUMERATION OF COLIFORM BACTERIA USING MULTIPLE TUBE FERMENTATION METHOD

Multiple fermentation tube technique

The technique has been used for the analysis of drinking-water for many years with satisfactory results. It is the only procedure that can be used if water samples are very turbid or if semi-solids such as sediments or sludges are to be analysed. The procedure followed is fundamental to bacteriological analyses and the test is used in many countries. It is customary to report the results of the multiple fermentation tube test for coliforms as a most probable number (MPN) index. This is an index of the number of coliform bacteria that, more probably than any other number, would give the results shown by the test. It is not a count of the actual number of indicator bacteria present in the sample.

Principle

Separate analyses are usually conducted on five portions of each of three serial dilutions of a water sample. The individual portions are used to inoculate tubes of culture medium that are then incubated at a standard temperature for a standard period of time. The presence of coliforms is indicated by turbidity in the culture medium, by a pH change and/or by the presence of gas. The MPN index is determined by comparing the pattern of positive results (the number of tubes showing growth at each dilution) with statistical tables. The tabulated value is reported as MPN per 100 ml of sample.

There are a number of variants to the multiple fermentation tube technique. The most common procedure is to process five aliquots of water from each of three consecutive 10-fold dilutions; for example, five aliquots of the sample itself, five of a 1/10 dilution of the sample and five of a 1/100 dilution.

Aliquots may be 1-ml volumes, each added to 10 ml of single-strength culture medium, or 10-ml volumes, each added to 10 ml of double-strength medium. Results are compared with values such as those given in Table. The use of one of the following variants of the technique may help to reduce the cost of analysis:

A smaller number of tubes is incubated at each dilution, for example three instead of five. A different table must then be used for the MPN determination (see Table 10.6 later). Some precision is lost, but using 9 tubes instead of 15 saves materials, space in the incubator, and the analyst’s time. •

For samples of drinking-water, one tube with 50 ml of sample and five tubes with 10 ml of sample are inoculated and incubated. The results are compared with the values such as those

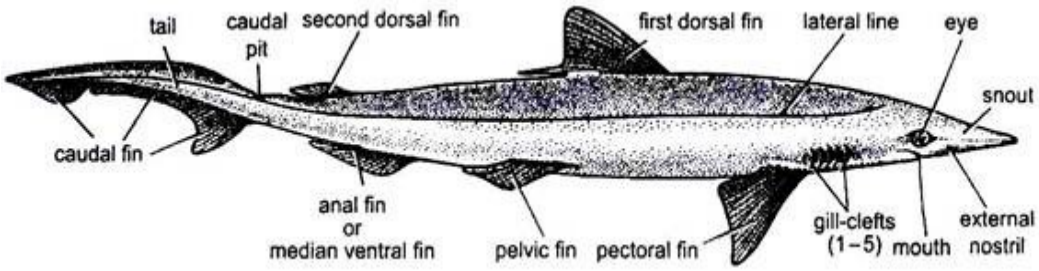
given in Table 10.7 (see later) to obtain the MPN. 10.3.1 Culture media and buffered dilution waterEach part of the test requires a different type of medium. For example, when enumerating coliforms, lauryl tryptose (lactose) broth is used in the first (isolation or presumptive) part of the test. In the second (confirmation) part, brilliant green lactose bile (BGLB) broth is used to confirm total coliforms and E. coli medium to confirm faecal coliforms. Some of the characteristics of these and other media suitable for use in most probable number analyses are described in Table 10.2. Media can be made from primary ingredients but are also available in the following forms: • Dehydrated powder, packaged in bulk (200 g or more), to be weighed out when the medium is prepared and dissolved in an appropriate volume of distilled water, dispensed to culture tubes and sterilised before use. • Dehydrated powder, packaged in pre-weighed amounts suitable for making one batch of medium, to be dissolved in an appropriate volume of distilled water, dispensed to culture tubes and sterilised before use. • Ampoules of solution, ready to use. The ampoules of ready-to-use media are the most convenient form but are the most expensive and have the shortest shelf-life. Pre-weighed packages are easy to use and reduce the risk of error in making up a batch of medium. Media are inevitably expensive when packaged in small quantities. However, they are not a major component of the cost of bacteriological analysis, and the extra cost may be negligible when compared with the greater convenience and reduced wastage. Large bottles containing dehydrated media must be tightly resealed after use to prevent spoilage; this is especially important in humid environments. Media should be stored in a cool, dark, dry place. After a medium has been prepared by dissolving the powder in distilled water, it should be distributed into culture tubes or bottles and sterilised. Batches of media should be tested before use, using a known positive and negative control organism. If the appropriate reactions are not observed, the media and the control organisms should be investigated and the tests repeated. Media should be used immediately but may be stored for several days provided that there is no risk of their becoming contaminated. A stock solution of buffered dilution water is prepared by dissolving 34.0 g of potassium dihydrogen phosphate, KH_2PO_4, in 500 ml of distilled water. The pH is checked and, if necessary, adjusted to 7.2 by the addition of small quantities of 1 mol l-1 NaOH solution. Distilled water is added to bring the final volume to 1 litre. The buffered water is stored in a tightly stoppered bottle in the refrigerator.

To prepare bottles of dilution water, 1.25 ml of stock solution is added to 1 litre of distilled water, mixed well and dispensed into dilution bottles in quantities that will provide, after

sterilisation, 9 or 90 ml. The bottles are loosely capped, placed in the autoclave, and sterilised for 20 minutes at 121 °C. After the bottles have been removed from the autoclave, the caps should be tightened and the bottles stored in a clean place until needed. Table 10.2 Culture media for most probable number (MPN) analyses MediumUsesIncubation temperatureRemarksIsolation mediaLactose broth Total or thermotolerant coliforms 48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms Prepare single strength medium by diluting double strength medium with distilled water. Each tube or bottle should contain an inverted fermentation (Durham) tube MacConkey broth Total or thermotolerant coliforms 48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms Improved formate lactose glutamate medium Total or thermotolerant coliforms 48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms Available commercially in dehydrated form as Minerals Modified Glutamate Medium Lauryl tryptose (lactose) broth Total or thermotolerant coliforms 48 hours at 35 ± 0.5 °C or 37 ± 0.5 °C for total coliforms and 24 hours at 44 ± 0.25 °C or 44.5 ± 0.25 °C for thermotolerant coliforms Confirmatory mediaBrilliant green lactose bile broth Total or thermotolerant coliforms (gas production) 44.5 ± 0.25 °C for thermotolerant coliforms EC medium Thermotolerant coliforms (indole production) 44.5 ± 0.25 °C for thermotolerant coliforms Addition of 1 % (m/m) L- or DL-tryptophan may improve performance of the medium Tryptone water Thermotolerant coliforms (gas + indole production) 44.5 ± 0.25 °C for thermotolerant coliforms Lauryl tryptose mannitol broth with tryptophan Thermotolerant coliforms (gas + indole production) 44.5 ± 0.25 °C for thermotolerant coliforms

Course No: ZOO 396A: Fishery Practical-I

Identification of fin fish, Ray fish,Shell fish. *Scoliodon sp.* (Indian shark/Dogfish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the lateral side of the body.
2. Pectoral fins are constricted at the base.

Hence, the specimen belongs to the Superorder Pleurotremata.

1. Presence of two dorsal fins with spines.
2. Presence of a single anal fin.
3. Presence of five gill-slits.

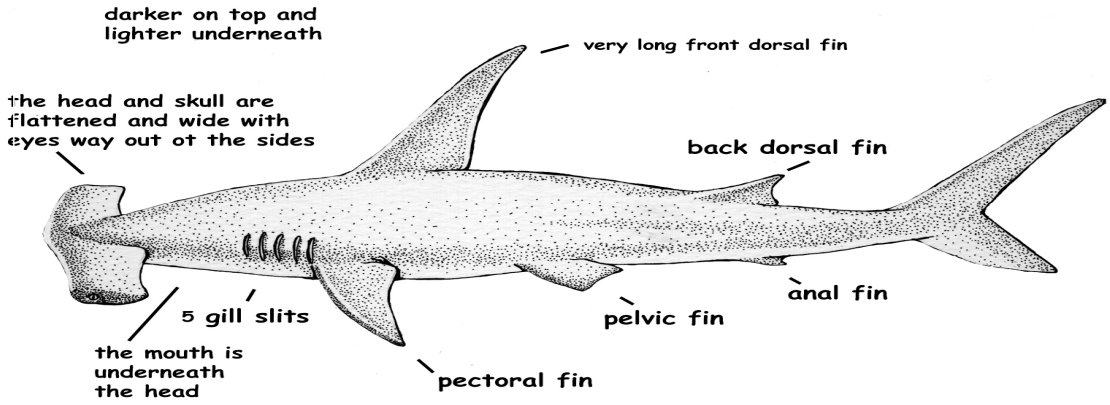
Hence, the specimen belongs to the Order Lamniformes.

1. Body is divisible into head thorax and abdomen.
2. Head is dorso-ventrally flattened but the rest of the body is laterally compressed.

3. Possess several sets of **homodont** teeth.
4. **Cloacal** aperture is longitudinal.

Hence, the specimen seems to be *Scoliodon sp.*

Sphyrna sp. (Hammer headed shark)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the lateral side of the body.
2. Pectoral fins are constricted at the base.

Hence, the specimen belongs to the Superorder Pleurotremata.

1. Presence of two dorsal fins with spines.
2. Presence of a single anal fin.
3. Presence of five gill-slits.

Hence, the specimen belongs to the Order Lamniformes.

1. **Malleate**-shaped head bearing eyes and nasal openings at the lateral extremities.

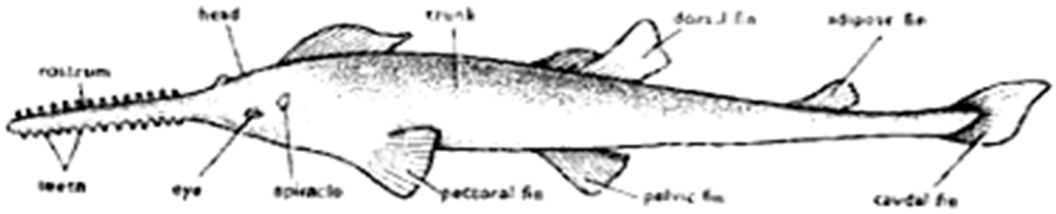
2. Eyes contain nictitating membrane.

3. **Cresent**-shaped ventrally located mouth.

4. Dorsal fins devoid of spines.

Hence, the specimen seems to be *Sphyrna sp.*

Pristis sp. (Saw fish)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.

2. Tail is **heterocercal.**

3. Presence of calcareous endoskeleton.

4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.

2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the ventral side of the body.

2. Pectoral fins are enlarged but tail and other fins are reduced.

Hence, the specimen belongs to the Superorder Hypotremata.

1. Body is fleshy and dorsoventrally compressed.

2. Eyes are on the dorsal surface.

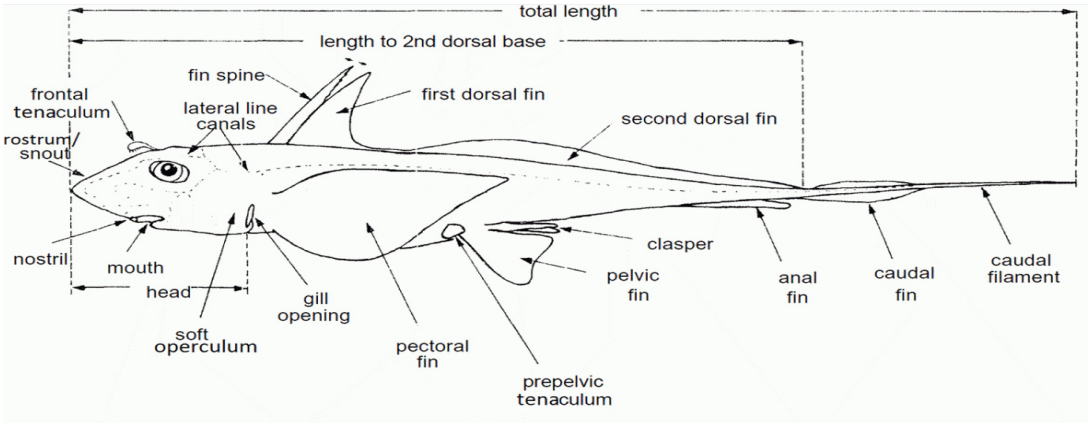
Hence, the specimen belongs to the Order Rajiformes.

1. Anterior part of the body is dorsoventrally flattened while the posterior part is shark like.

2. **Snout** is prolonged into a long **rostrum** with a series of lateral tooth-like **denticles.**

Hence, the specimen seems to be *Pristis sp.*

Hydrolagus sp. (Ghost shark) (Chimaera)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. The tail is long and thin and they move by sweeping movements of the large pectoral fins.
2. The erectile spine in front of the dorsal fin is sometimes venomous.
3. The mouth is a small aperture surrounded by **lips**, giving the head a **parrot-like** appearance.

Hence, the specimen belongs to the Subclass Holocephali.

1. Elongated, soft bodies, with a bulky head and a single gill-opening.
2. Have a **venomous spine** in front of the dorsal fin.
3. Male chimaeras have retractable sexual appendages on the forehead and in front of the pelvic fins.
4. Their upper jaws are fused with their skulls having three pairs of large permanent grinding tooth plates.
5. Have gill covers or **opercula** like bony fishes.

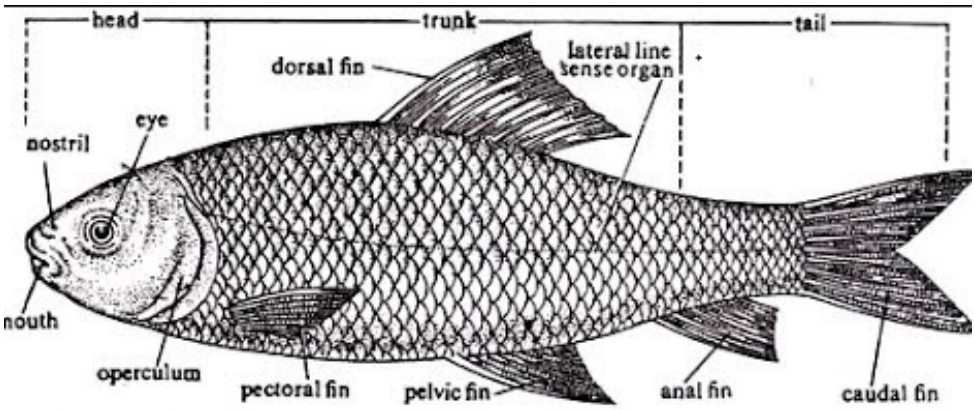
Hence, the specimen belongs to the Order Chimaeriformes.

1. Have a smooth and scaleless skin that is a silvery-bronze color, often with sparkling shades of gold, blue, and green.

2. Dark edges outline both the caudal and dorsal fins, whereas the pectoral fins have a transparent outline.
3. Pectoral fins are large and triangular, and extend straight out from the sides of their bodies like airplane wings.
4. It has a duckbill-shaped snout and a rabbit-like face.
5. Have large, emerald green eyes, which are able to reflect light.

Hence, the specimen seems to be *Hydrolagus sp.*

Labeo sp. (Rohu)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**

2. Tail is **homocercal.**

3. Presence of bony endoskeleton.

4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.

2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.

2. Gills are fully filamentous.

3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. No scale on the head, head is covered by plates.

2. No teeth in the jaws.

3. Single large dorsal fin.

4. Fins supported by soft fin rays.

5. Lateral line distinct.

Hence, the specimen belongs to the Order Cypriniformes.

1. Body covered with large cycloid scales, no scale on the head.

2. Head prominent with blunt snout projecting beyond the narrow mouth.

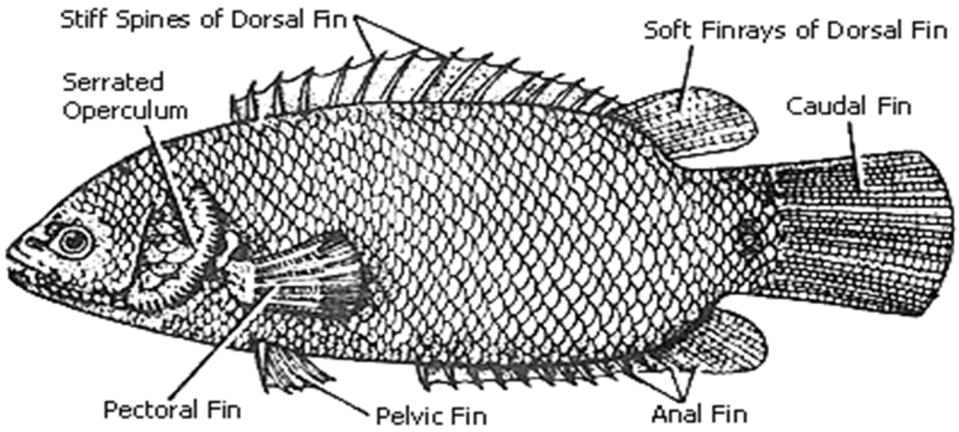
3. Lips thick and horny covering the jaw, having inferior transverse fold.

4. Barbels are very short or absent.

5. Body colour is bluish-brown on the dorsal aspect, and silvery-white with reddish-orange tinge on the ventral aspect.

Hence, the specimen seems to be *Labeo sp.*

Anabus sp. (Koi fish)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**

2. Tail is **homocercal.**

3. Presence of bony endoskeleton.

4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.

2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.

2. Gills are fully filamentous.

3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Dorsal and anal fins are both with spiny and soft rays.

2. Dorsal fins are two in number.

3. Body is covered by **ctenoid** scales.

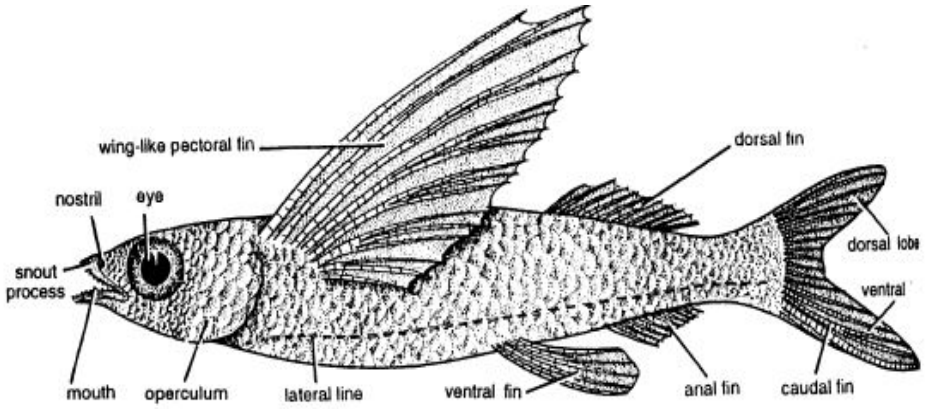
Hence, the specimen belongs to the Order Perciformes.

1. Dorsal and anal fins are divided and supported by anterior spiny and posterior soft rays.

2. Small spiny projections are present along the posterior edge of operculum.

Hence, the specimen seems to be *Anabus sp.*

Exocoetus sp. (Flying fish)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**

2. Tail is **homocercal.**

3. Presence of bony endoskeleton.

4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.

2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.

2. Gills are fully filamentous.

3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Actually spindle like long body.

2. Pectoral fins are very big and modified into flying structure.

3. Pelvic fins are at the abdominal region.

Hence, the specimen belongs to the Order Beloniformes.

1. Pectoral fins are enormously elongated to form wing-like structures. They serve as parachute to sustain the fish in its gliding leap.

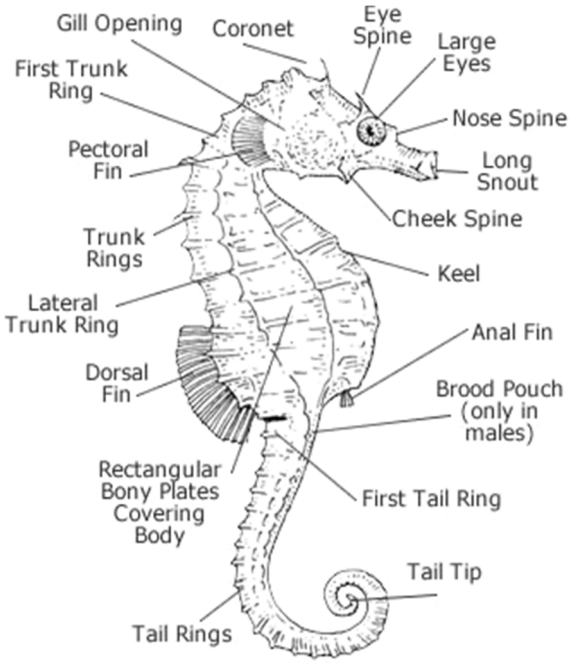
2. Lateral line is located low on the body.

3. Pectoral fin with black spots.

4. Tail is hypobatic and ventral lobe of the tail fin is much elongated and help in skipping over the water.

Hence, the specimen seems to be *Exocoetus sp.*

Hippocampus sp. (Sea horse)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**

2. Tail is **homocercal.**

3. Presence of bony endoskeleton.

4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.

2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.

2. Gills are fully filamentous.

3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Body is covered by ring like bony plates.

2. Snout tubular with suctorial mouth.

Hence, the specimen belongs to the Order Solenichthyes.

1. Head is at right angle to the body axis.

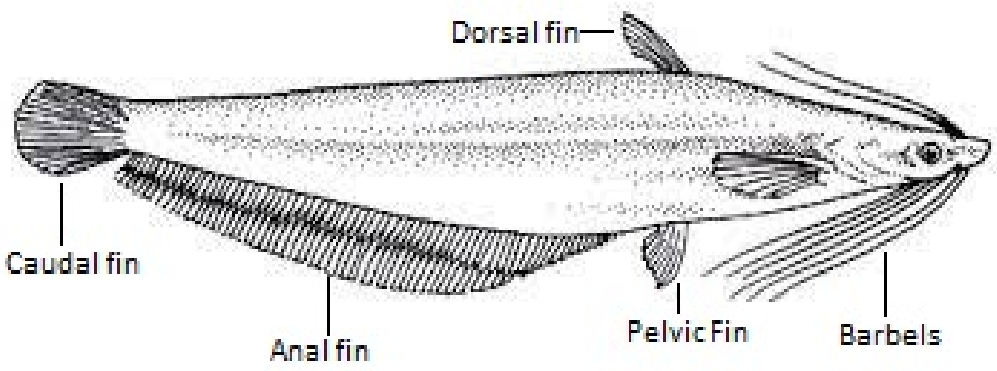
2. Pectoral fin is transparent behind the operculum.

3. Tail is long and **prehensile.**

4. Presence of **brood pouch** on the belly (in male)/an anal fin on the belly (in female).

Hence, the specimen seems to be *Hippocampus sp.*

Heteropneustes sp. (Singhi)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**

2. Tail is **homocercal.**

3. Presence of bony endoskeleton.

4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.

2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.

2. Gills are fully filamentous.

3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Pectoral fin with a spine.

2. Barbels present.

3. Scales absent.

Hence, the specimen belongs to the Order Siluriformes.

1. Skin is scaleless.

2. Head is flat with four pairs of **barbells.**

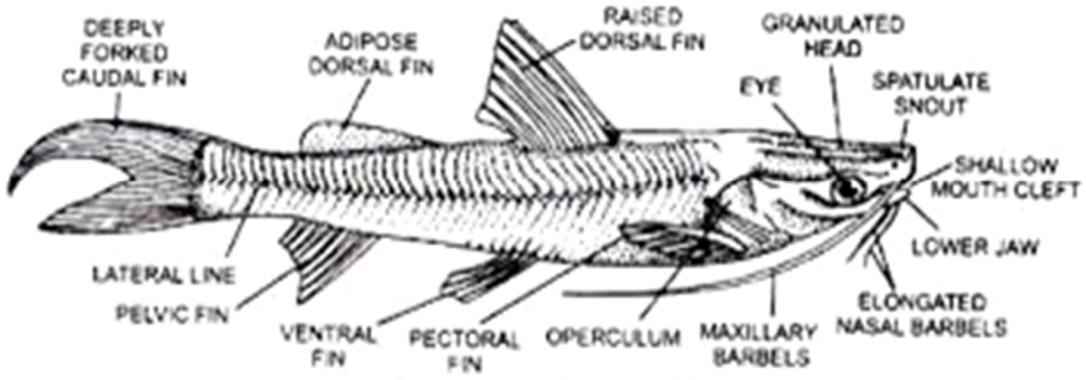
3. First ray of pelvic fin is modified into a serrated spine.

4. Dorsal fin is very small.

5. Caudal fin is separated by a notch from extended anal fin.

Hence, the specimen seems to be *Heteropneustes sp.*

Mystus sp. (Tangra)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Pectoral fin with a spine.

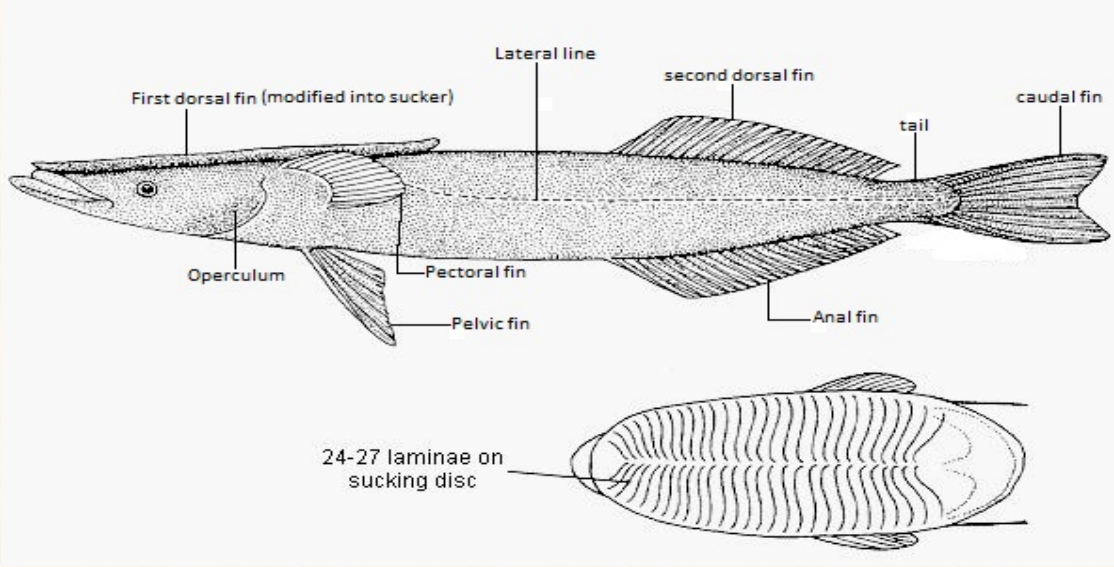
2. Barbels present.
3. Scales absent.

Hence, the specimen belongs to the Order Siluriformes.

1. Presence of **adipose** dorsal fin.
2. Body is blackish above and slivery on the sides.
3. Snout is distinctly long with four pairs of barbells.
4. Presence of light and dark coloured longitudinal bands on bodywall.

Hence, the specimen seems to be *Mystus sp.*

Echeneis sp. (Sucker fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Body covered by cycloid scales.

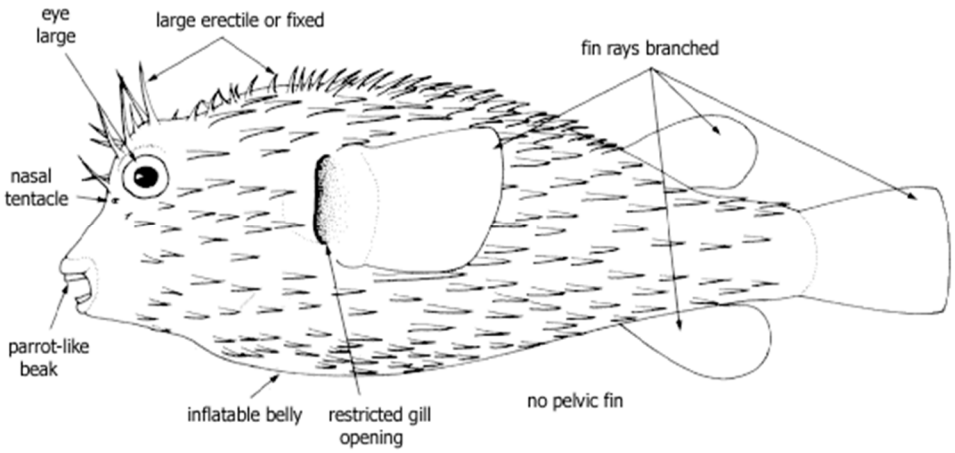
2. Swim bladder absent.
3. First dorsal fin modified into sucker on the dorsal side of the head.
4. Fins are without rays.

Hence, the specimen belongs to the Order Echeniformes.

1. First dorsal fin modified into sucker.
2. Dorsal and anal fins without rays.
3. Dorsal and anal fins are identical and opposite to each other.

Hence, the specimen seems to be *Echeneis sp.*

Diodon sp. (Porcupine fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

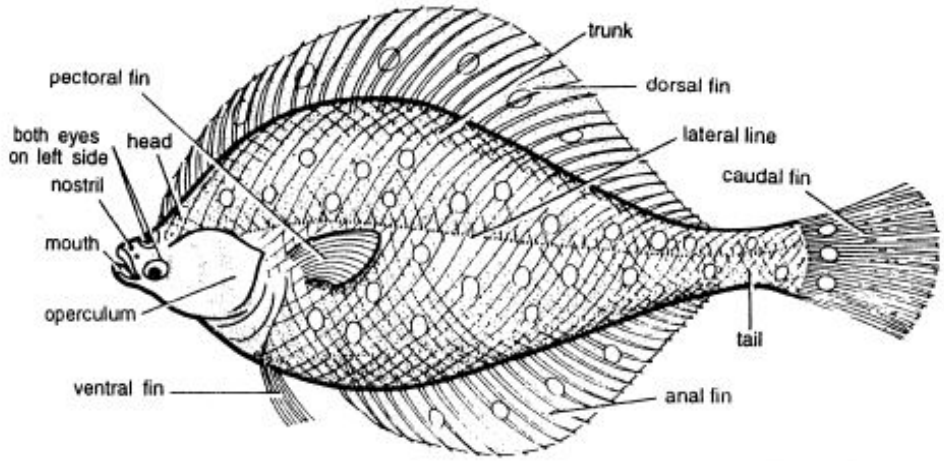
1. Range from nearly square or triangular, globose to laterally compressed.
2. The bones of the jaw are modified and fused into a sort of "beak"; visible sutures divide the beaks into "teeth".

Hence, the specimen belongs to the Order Tetraodontiformes.

1. Jaws are aided by powerful muscles, and many species also have pharyngeal teeth to further process prey items.
2. They lack swim bladders and spines, and are propelled by their very tall dorsal and anal fins.
3. The caudal peduncle is absent and the caudal fin is reduced to a stiff rudder-like structure.

Hence, the specimen seems to be *Diodon sp.*

Pleuronectes sp. (Flat fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

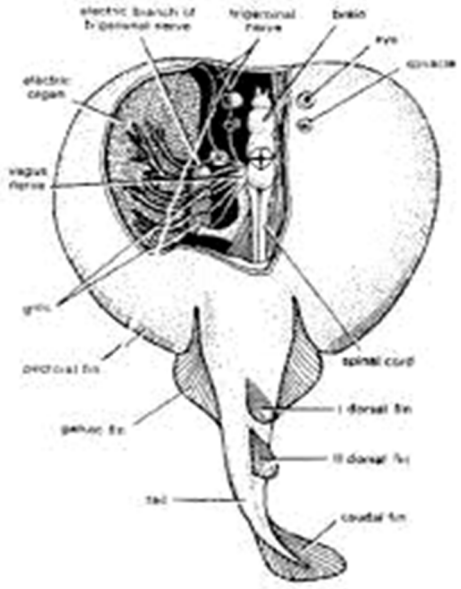
1. Body highly compressed, somewhat rounded on eyed side and flat on blind side.
2. Asymmetrical body, with both eyes lying on the same side of the head in the adult fish.
3. Dorsal and anal fins with long bases.

Hence, the specimen belongs to the Order Pleuronectiformes.

1. The surface of the fish facing away from the sea floor is pigmented.
2. Protruding eyes above body surface.
3. Presence of six or seven branchiostegal rays.
4. Adults having small body cavity; swim bladder absent.
5. Scales **cycloid, ctenoid** or **tuberculate**.

Hence, the specimen seems to be *Pleuronectes sp.*

Torpedo sp. (Electric Ray)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the ventral side of the body.
2. Pectoral fins are enlarged but tail and other fins are reduced.

Hence, the specimen belongs to the Superorder Hypotremata.

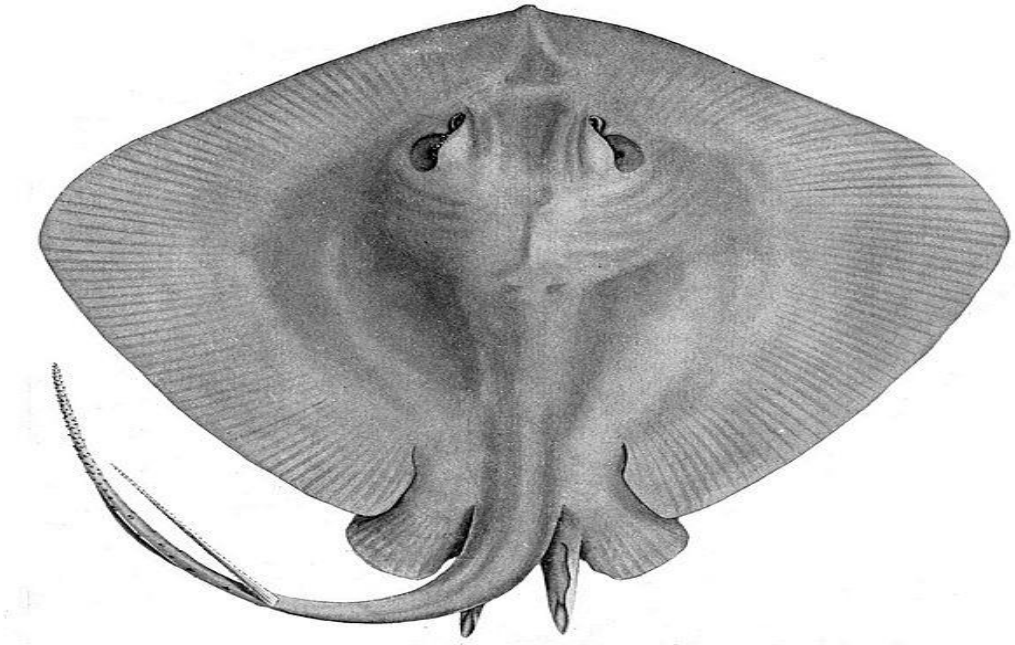
1. Presence of electric organ between head and pectoral fin.

Hence, the specimen belongs to the Order Torpediniformes.

1. Anterior end of the body is semi-circular.
2. Mouth is a slit-like aperture.
3. Tail is short and thick with two dorsal fins and a caudal fin.

Hence, the specimen seems to be *Torpedo sp.*

***Dasyatis* sp.**



Identifying Character:

- All members possess either in adult or larval stage a notochord, which forms the axialskeleton of the body
- Pharyngeal gill slits are present in all members

Hence, Phylum – Chordata

- Notochord is replaced by vertebral column
- Brain is well developed with brain box or cranium
- Gills are present as primary respiratory organ

Hence, Subphylum – Vertebrata

- Mouth bounded with jaws

Hence, Super class – Gnathostomata

- Skeletons made of cartilage rather than bone.
- Chondrichthyans have tough skin covered with dermal teeth.
- Chondrichthyans have toothlike scales called dermal denticles or placoid scales.
- All chondrichthyans breathe through five to seven pairs of gills, depending on the species.

Hence, Class - Chondrichthyes

- The disc is strongly depressed and varies from oval longitudinally to much broader thanlong;
- the tail is well marked off from the body sector, very short to long and whiplike, andequipped with a poisonous spine in some species;
- the pectoral rays are either continuous along the side of the head or separate from the headand modified to form rostral lobes or finlike rostral appendages (cephalic fins);
- The dorsal fin, if present, is near the base of the tail; and development is ovoviviparous.
- Sometimes an individual ray may have two or three, very rarely four, tail spines rather thanthe usual one.

Hence, Order – Myliobatiformes

- Medium to large rays (disc width to 2.1 m), the disc rhomboid to oval, its width ranging from greater to less than its length;
- snout obtuse and little produced to acute and moderately produced, head not elevated from disc; tail distinct from disc, slender and whip-like, equal to or much longer than distance from snout to cloaca, with one or several serrated spines on top near base, keels or membranous folds along upper or lower sides (or both) present in some species.
- Nostrils separated from mouth, but front margins greatly expanded to reach back and join each other. Mouth almost straight or arched, with a transverse row of

bulbous papillae alongfloor, teeth small and numerous.

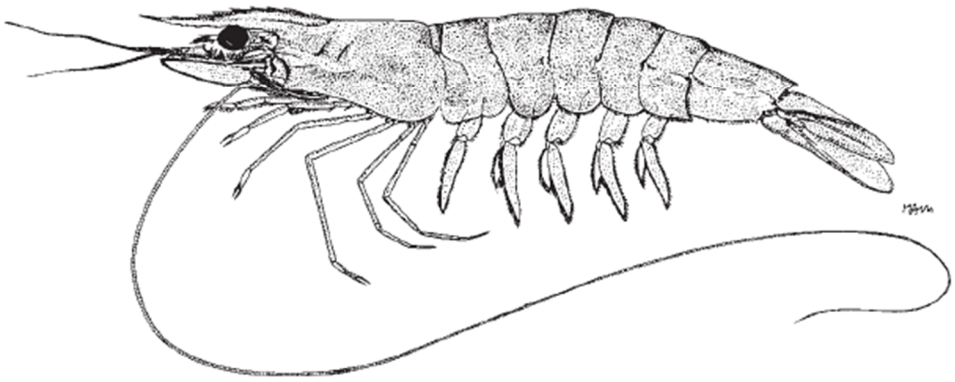
- Spiracles well developed, without tentacle-like processes. Dorsal and caudal fins absent, pectoral fins joined to side of head, the finrays beginning at tip of snout. Upper surfaces naked or covered with tubercles, thorns or thornlets.
- Benthic on soft bottoms, generally in shallow tropical and warm temperate waters, but also to depths of 200 m.

Hence, Family – Dasyatidae

- Snout obtuse and little produced to acute and strongly produced;
- disc more or less rhomboid, but not circular; tail whip-like,
- Filamentous near tip, with or without a membranous fold or ridge above, but with a foldbelow (beginning at level of spine).
- Upper surfaces with or without bucklers, tubercles and thorns with conical cusps.

Hence, Specimen – *Dasyatis* sp.

***Penaeus* sp.**



Identifying Character:

1. Body is segmented and covered by chitinous cuticle.
2. Body segments bear paired externally jointed appendages.
3. Usually with anterior photoreceptors.

Hence, the specimen belongs to Phylum Arthropoda.

1. Body is divided into two or three parts.

2. Head bears on or two pairs of antennae, two pairs of maxillae and one pair of mandible.
3. Usually compound eyes are present.

Hence, the specimen belongs to Subphylum Mandibulata.

1. Dorsal surface is covered by a carapace.
2. Head bears two pairs of preoral and three pairs of postoral appendages.
3. Antennules are uniramous while other appendages are biramous.
4. Biramous appendages are either **phyllopodium** or **stenopodium.**

Hence, the specimen belongs to Class Crustacea.

1. Body made up of 19 segments: 5 in head,8 in thorax and 6 in abdomen.
2. Presence of cephalothoracic carapace.
3.Appendages , on the sixth abdominal segment are flattened to form uropods.
4.Caudal style absent.

Hence, the specimen belongs to Subclass Malacostraca.

1 .Large carapace , fused with all thoracic segments.
2. Stalked eyes.

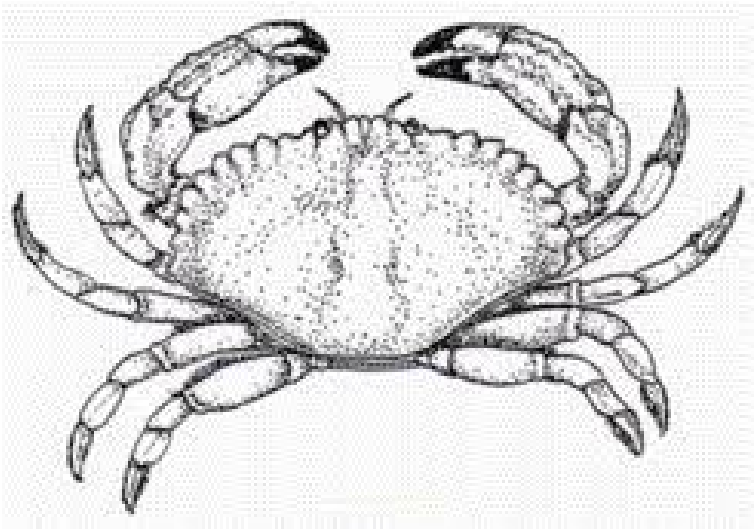
Hence, the specimen belongs to Suborder Eucarida.

1. First three pairs of thoracic appendages are maxillipedes and rest five pairs are walking legs.

Hence, the specimen belongs to order Decapoda.

1. Rostrum lacks ventral teeth.
2.Well developed third walking legs.
3.Rostral crest non-triangular in profile.
4.Whitish targa lacks black spot.

. Hence, the specimen seems to be *Penaeus* sp.



Identifying Character:

1. Body is segmented and covered by chitinous cuticle.
2. Body segments bear paired externally jointed appendages.
3. Usually with anterior photoreceptors.

Hence, the specimen belongs to Phylum Arthropoda.

1. Body is divided into two or three parts.
2. Head bears on or two pairs of antennae, two pairs of maxillae and one pair of mandible.
3. Usually compound eyes are present.

Hence, the specimen belongs to Subphylum Mandibulata.

1. Dorsal surface is covered by a carapace.
2. Head bears two pairs of preoral and three pairs of postoral appendages.
3. Antennules are uniramous while other appendages are biramous.
4. Biramous appendages are either **phyllopodium** or **stenopodium.**

Hence, the specimen belongs to Class Crustacea.

1. Body made up of 19 segments: 5 in head,8 in thorax and 6 in abdomen.
2. Presence of cephalothoracic carapace.
3.Appendages , on the sixth abdominal segment are flattened to form uropods.
4.Caudal style absent.

Hence, the specimen belongs to Subclass Malacostraca.

1 .Large carapace , fused with all thoracic segments.
2. Stalked eyes.

Hence, the specimen belongs to Suborder Eucarida.

1. First three pairs of thoracic appendages are maxillipedes and rest five pairs are walking legs.

Hence, the specimen belongs to order Decapoda.

1.Carapace oval and broader than long.

2.Small abdomen permanently fixed under the cephalothorax.

3.Five pairs of thoracic legs are well developed.

4. Rostrum and uropod absent.

Hence, the specimen seems to be *Scylla* sp (Crab).

2.. Qualitative and Quantitative estimation of zooplankton.

Introduction

The term *plankton* was first used by German biologist Victor Hensen in 1887. According to him plankton includes all organic particles which drift freely and involuntarily in open water, independent of shores and bottom. They have either relatively small power of locomotion or not at all. Plankton differs greatly in size and species.

Classification

Plankton can be broadly classified into 2 groups-

1. **Phytoplankton**- Theyincludes plants. Most of the phytoplankton survives on the open surface waters of lakes, rivers and oceans. Its community comprises of algal members from both prokaryotes and eukaryotes. It includes Cyanobacteria, Chlorophyta, Euglenophyta, Cryptophyta etc.

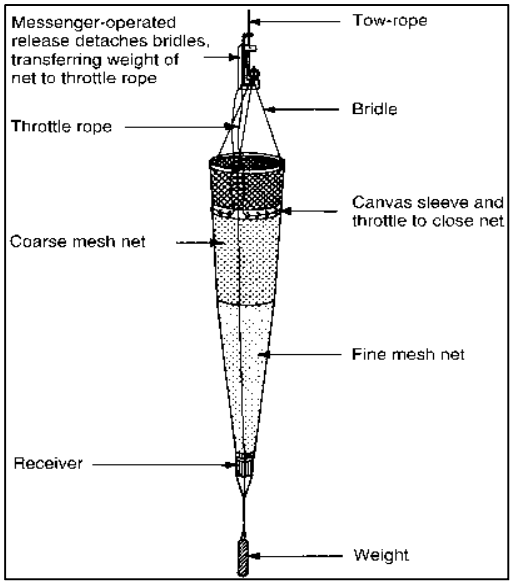
2. **Zooplankton**- They includes animals. Zooplanktonic population of a fresh water biota may be composed of Arthropods like cladocerans, copepods and ostracods along with pseudocoelomates like rotifers.

Plankton Net

Plankton nets are the most popularly used device for sampling and may vary in design that range from basic to tow nets to more complicated device fitted apparatus for more specific sample collection. Nets permit quantitative studies, since the mesh size will select the type of plankton collected. Sampling by nets is highly selective, depending on mesh size of the gauze, net towing speed and the species present in the water.

Structure of plankton net-A typical plankton net usable in the surface layers is conical in shape and has the following constituents:

- A net ring made up of stainless steel, wrapped and sealed with a polythene tude, present anteriorly.
- A non-filtering portion made of coarse khaki cloth is attached using button and hole system.
- The filtering portion is made of monofilament nylon material.



The determination of the volume of water filtered through any plankton net is essential for the estimation.

Materials required

Plankton net, 70% ethanol, plastic bucket, Sample bottle, Microscope, slide, cover slip, dropper and Sedgwick-Rafter Cell.

Procedure

a. *Collection and Preservation of planktons*

1. Planktons can be collected by filtering a known quantity of water through plankton net No. 25, mesh size 55µm. The surface water for this purpose may be collected with the aid of a plastic bucket of specific volume.

2. Plankton net weighted at the base is lowered into the water to known depth with the help of a graduated rope. The net is then pulled gradually out. The volume of water filtered through the plankton net is calculated

$$V=\pi r^2$$

Where, ***V****= volume of water (L)*
π*= 3.142*
r*= radius of mouth of plankton net (m)*
h*= depth up to which the net was lowered*

Plankton collected is transferred to a small sample bottle (50-100 ml) and preserved in 70% ethanol.

b. ***Quantitative analysis of plankton population*** **(Sedgewick Rafter Method)**

1. Concentrated sample is shaken well and without delay, 1 ml of it is transferred in the cavity of Sedgewick Rafter cell with a dropper.
2. Cavity of the cell is covered with a cover slip avoiding any air bubble to get in. planktons are allowed to settle and then counted under a microscope.
3. At least 10 replicates are taken and the average count per ml is calculated.

Calculation

$$\textbf{Zooplankton (Units/L)} = \frac{\textbf{N X C}}{\textbf{Volume of sample in L}}$$

Where, **N**= number of plankton counted in 1 ml concentrate
C= total volume of concentrate (ml)
Volume of sample in L= total volume of water taken

c. ***Qualitative analysis of plankton population***

Each type of plankton is separated by sieving and then microscopic observation of the planktons is done for Genus and species identification. Separated planktons are preserved in 70% alcohol.

Identification-

Pelagic Community-
Zoolplankton

1. ***Daphnia* sp.**
Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Branchiopoda
Order-Cladocera
Type-*Daphnia*

Distinctive features
1. Bilaterally compressed body, enclosed in a transparent bivalve carapace, ending into sharp caudal spine and anteriorly into pointed rostrum.
2. Rounded head bears large **biramous antennae**.
3. Large, sessile, single, huge eye is very distinct.
4. Five pairs of leaf like thoracic appendages and abdomen devoid of appendages.
5. In females, there is a brood pouch near the back.

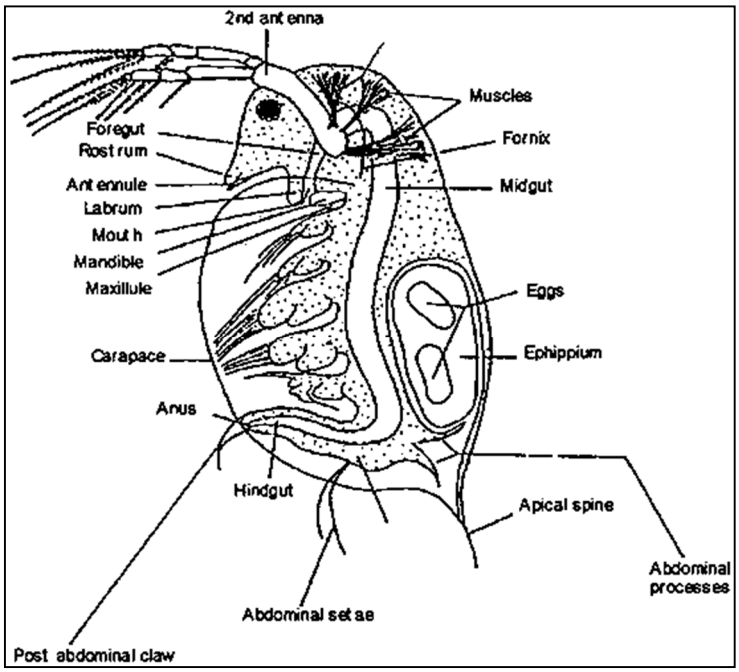


Fig 1.*Daphnia* sp.

Ecological comments

Daphnia often plays the role of keystone species in ponds and lakes, where they are the most important primary consumer, filter-feeding small suspended particles, particularly

algae. They also play an important role in aquatic food webs by being the food reserve for fish and other invertebrate predators.

2. ***Moina* sp.**
Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Branchiopoda
Order-Anomopoda
Type-*Moina*

Distinctive features
1. Body oval shaped.
2. Antennae are branched.
3. Rostrum is in horizontal arrangement.
4. Body terminates into a pair of caudal styles.

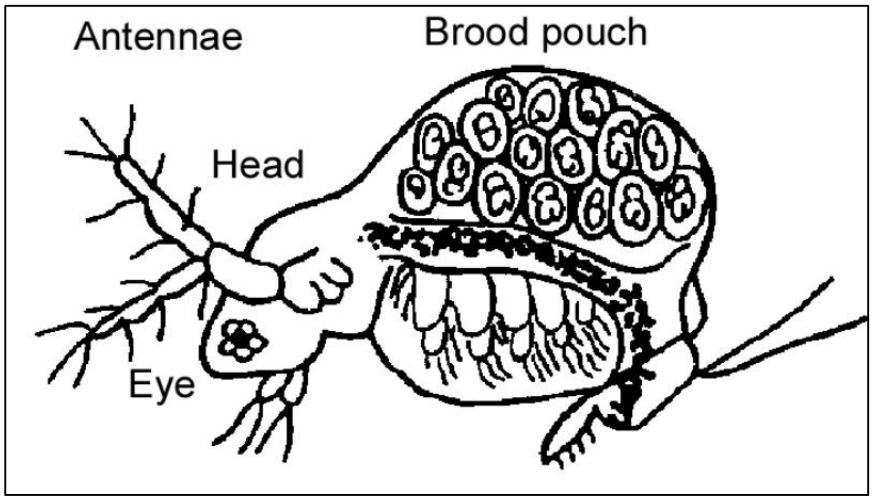


Fig 2.*Moina*sp.

Ecological comments

Moina feed on various groups of bacteria, yeast, phytoplankton and detritus and they are live food for finfish, crustaceans, teleost and marine fishes because of their high protein and nutrient content thus maintaining the aquatic food web. They are often used a feed in aquaculture thus having a high economic value.

3. ***Cyclops* sp.**

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata

Class-Crustacea
Subclass-Copepoda
Order-Cyclopoida
Type-*Cyclops*

Distinctive features
1. Pear shaped body with broad anterior and narrow posterior end.
2. The head and first thoracic segment fuse to form cephalothorax.
3. A median eye is present near the anterior end.

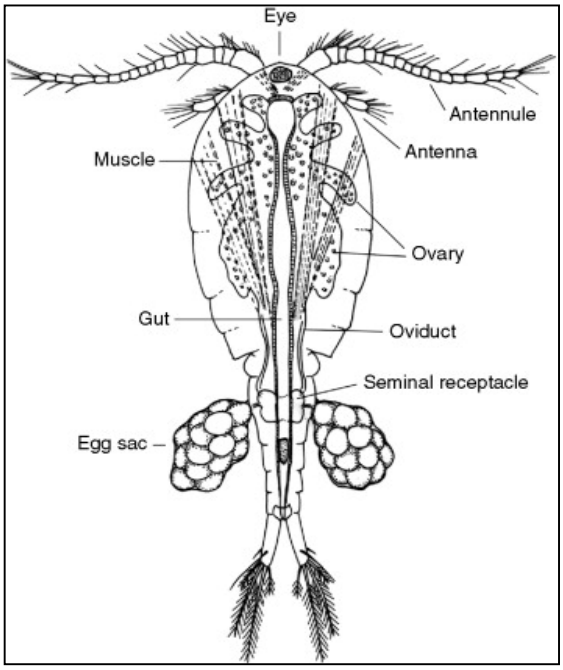


Fig 3.*Cyclops* sp.

Ecological comments

Cyclops play a bridging role in the aquatic food web by consuming dinoflagellates and phytoplanktons thus providing food sources for higher level predatory nekton species. It is the intermediate host of guinea-worm disease and fish tapeworm infection and can be passed to humans through infected water consumption.

4. ***Cypris* sp.**

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Ostracoda
Type-*Cypris*

Distinctive features
1. The body is entirely enclosed within a bivalve carapace.
2. Well developed antennae used in swimming.

3. Gills enclosed within the valves.
4. Head bears four pairs of appendages.
5. Presence of three pairs of thoracic appendages.

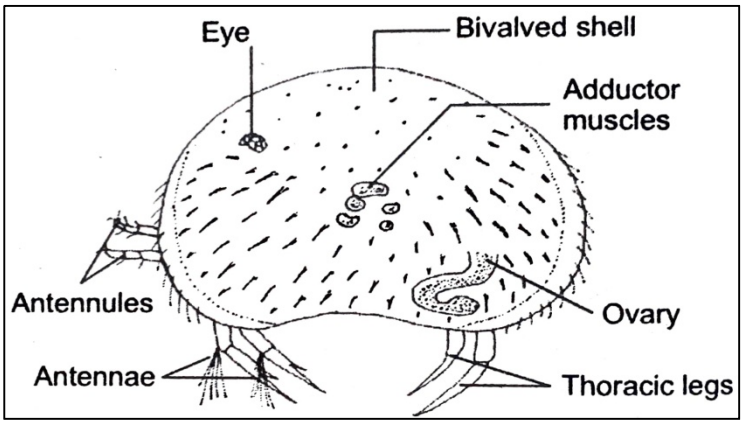


Fig 4.*Cypris* sp.

Ecological comments

Cypris has an important role in the aquatic ecosystem serving as vital food sources for larger aquatic predators like fish, other crustaceans, benthic molluscs etc. They can also recycle nutrients as filter feeders

5. ***Nauplius*larvae**

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Type-*Nauplius*

Distinctive features
1. Minute, conical and microscopic with broad anterior and narrow posterior end.
2. Body is oval and divided into indistinct head, trunk and anal region.
3. It contains single median eye and frontal and lateral horns.
4. Three pairs of appendages namely uniramous antennules, biramous antennae and biramous mandibles.
5. Larvae contain mouth and gut.

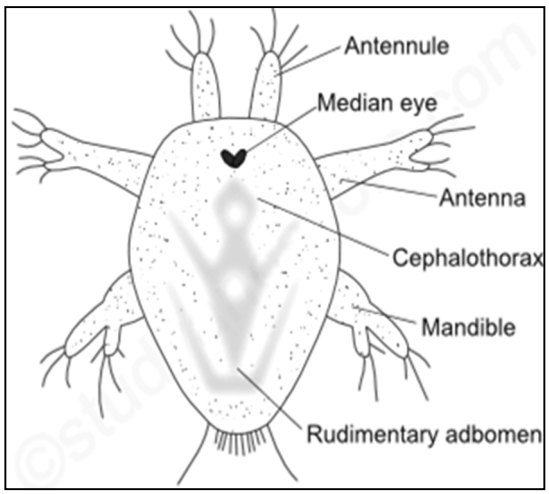


Fig 5. Nauplius larva of *Cyclops* sp.

Ecological comments

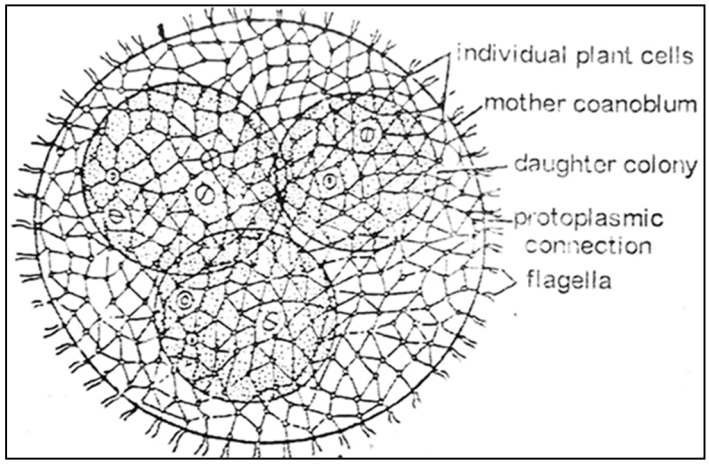
The Naplius larvae sometimes functions as food gatherers and has a well-developed alimentary system storing the food so that the transformation to the adult stage can occur.

6. ***Volvox* sp. (Phytoplankton/Periphyton)**

Systematic Position
Division- Chlorophyta
Class-Chlorophyceae

Distinctive features
1. Free floating fresh water green algae.
2. Each mature *Volvox* colony is composed of up to thousands of cells from two differentiated cell types.
3. Adult somatic cells comprise a single layer with the flagella facing outward.
4. The cells have anterior eyespots that enable the colony to swim toward light.
5. *Volvox* is facultatively sexual and can reproduce both sexually and asexually.

Fig 6.*Volvox* sp.



Ecological comments

They can be found in ponds and other fresh water bodies throughout the world. They contribute to the production of oxygen and serve as food for a number of aquatic organisms like rotifers.

Benthic Community-

1. ***Ephemera* sp.** (May flylarvae)

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Insecta
Subclass- Pterygota
Order-Ephemeroptera
Type-*Ephemera*

Distinctive features
1. Sides of the abdomen with plate like, feather like or leaf like gills, usually with three terminal filaments.
2. Tarsal claw single.
3. Older larvae show developing wing pads.
4. Gills feathery, consists of two branchiaes each thickly fringed with filaments.
5. Gills held over back during life.
6. Mandibles project beyond front of the head.

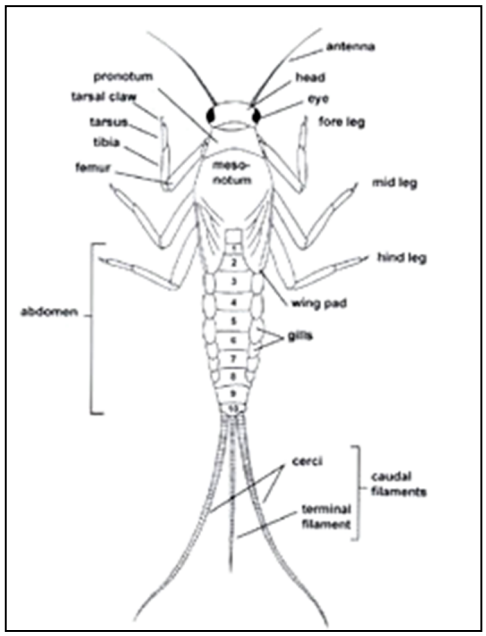


Fig 7.*Ephemera* sp. (May flylarvae)

Ecological comments

Mayflies are especially important to fishing contributing to the provisioning services of ecosystems in that they are utilized as food by human cultures worldwide, as laboratory and as potential source of anti tumour molecules.

2.**Dragon fly nymph**

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Insecta
Subclass- Pterygota
Order-Odonata
Type-Dragon fly nymph

Distinctive features
1. Labium prominent, extensible forming a food capturing structure larger than head.

2. Abdomen terminating in three to five stiff pointed valves, longest less than one third of abdomen.

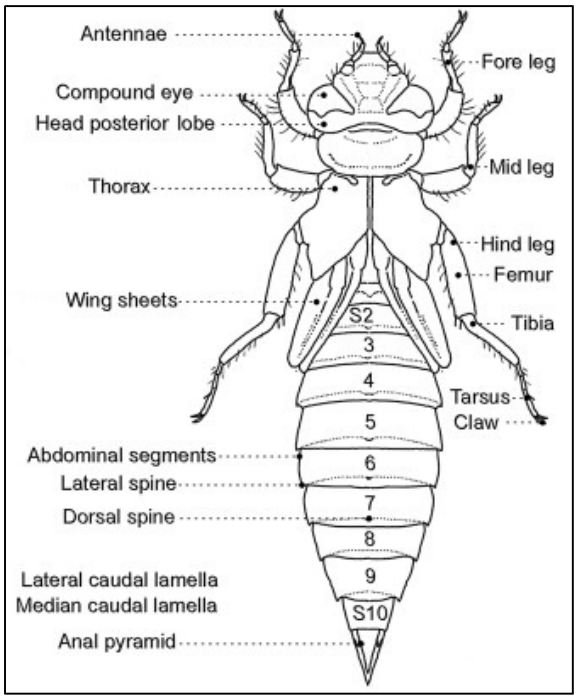


Fig 8. Dragon fly nymph

Ecological comments

They are freshwater insects showing semi aquatic life cycle. They also play an important role in controlling other disease spreading insect population such as mosquitoes acting as underwater predators.

Aquatic insect-

1. ***Belostoma* sp.** (Giant water bug)

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Insecta
Subclass- Pterygota
Order-Hemiptera
Type-*Belostoma*

Distinctive features
1. Large insect with elongated body covered by leathery brownish exoskeleton.
2. Mouth parts piercing and sucking type.
3. Legs flat, forelegs shorts and raptorial while posterior legs adopted for swimming and crawling.
4. Thorax broad and somewhat triangular.

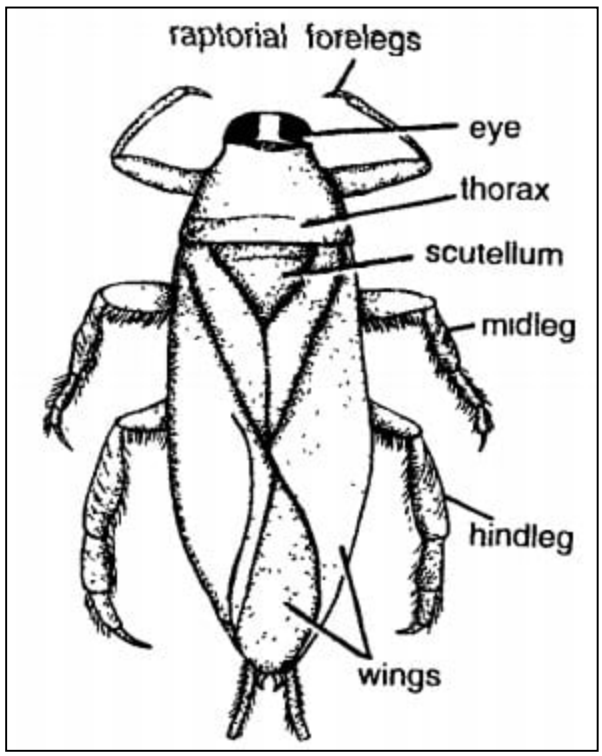


Fig 9.*Belostoma* sp.

Ecological comments

Belostoma inhabit permanent lentic habitats, especially weedy ponds, margins of lakes and marshes. They are important as apex invertebrate predators.

3.Identification of fish parasites.

Subclass / family	Genus	Characteristics
Branchiura		
Argulidae	*Argulus* sp.	Body broad and flat covered anteriorly with dorsal shield with a pair of compound eyes,hooks and barbs,which it uses to attach to the fins, gills and skin of its host, second maxillae usually form prehensile suckers.
Copepoda		
Caligidae	*Caligus* sp. *Lepeophtheirus* sp. *Anuretes* sp	Transparent, cephalothorax covered dorsally by a sub circular shield, with a pair of suckers on the frontal edge of the body and four pair of legs,vestigial abdomen in some species, found in large numbers on gills and body surface with different stages of life cycle in the same host
Ergasilidae	*Ergasilus* sp.	Cephalothorax constituting half or more of body length, the second antennae are modified for clinging to the host, moderate to large numbers on gills with rigorous feeding action and movements
Lernanthropidae	*Lernanthropus* sp.	Few in number but large in size, feed on gill tissues and blood, seriously damage the tissues
Lernaeidae	*Lernaea* sp	Body unsegmented, with its anterior part deeply embedded in host tissue with the help of a hold fast organ,infect nostril, skin, fin, gills, buccal cavity
Isopoda		
Cymothoidae	*Cymothoa* sp.	Entire dorsal surface of body divided into many narrow segments, eyes are sessile, parasite immovably attached to surface, buccal or branchial cavity of fish.

4.ARO in fishes.

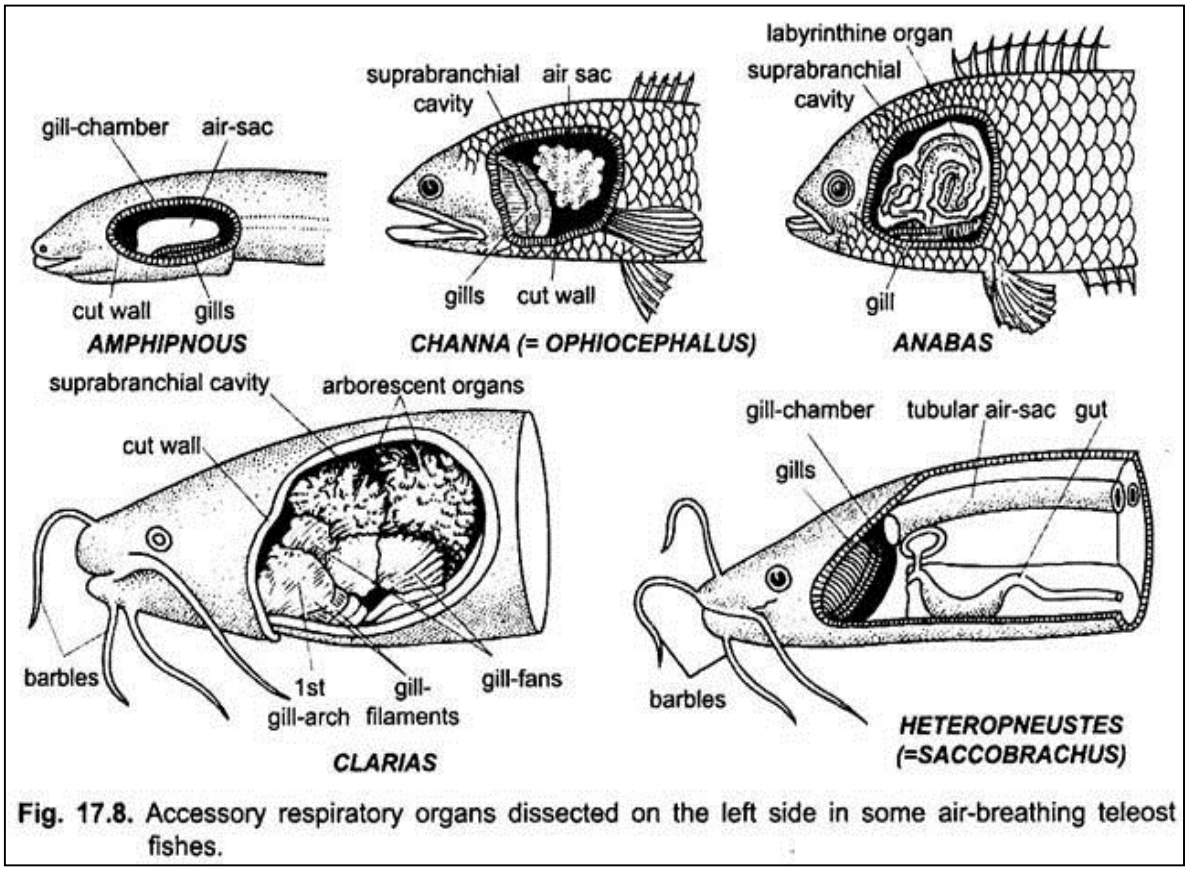
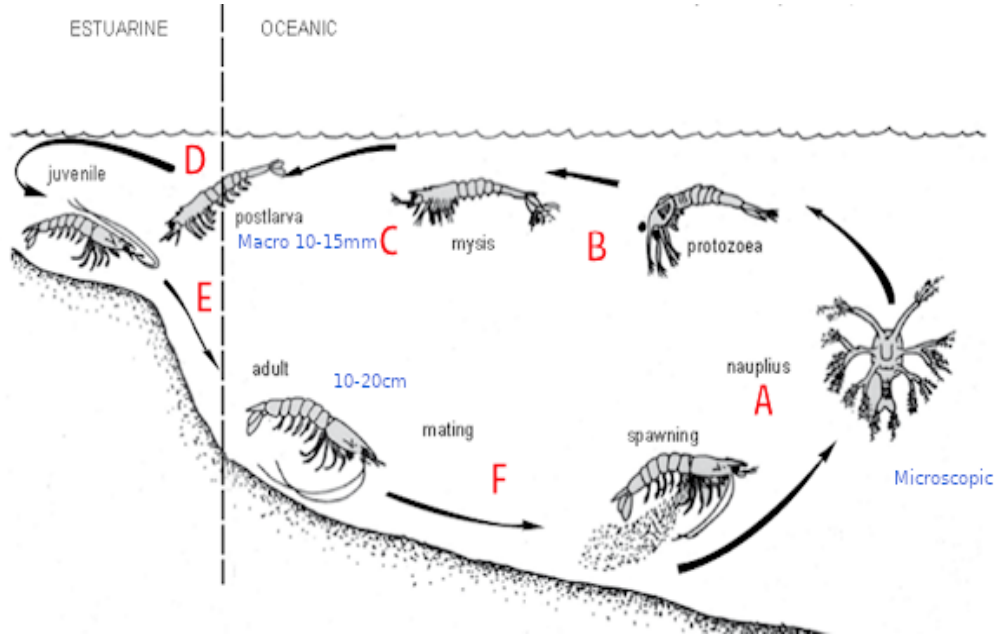


Fig. 17.8. Accessory respiratory organs dissected on the left side in some air-breathing teleost fishes.

6. Identification of different development stages of prawns and crabs.

- **Developmental stages of Prawn**



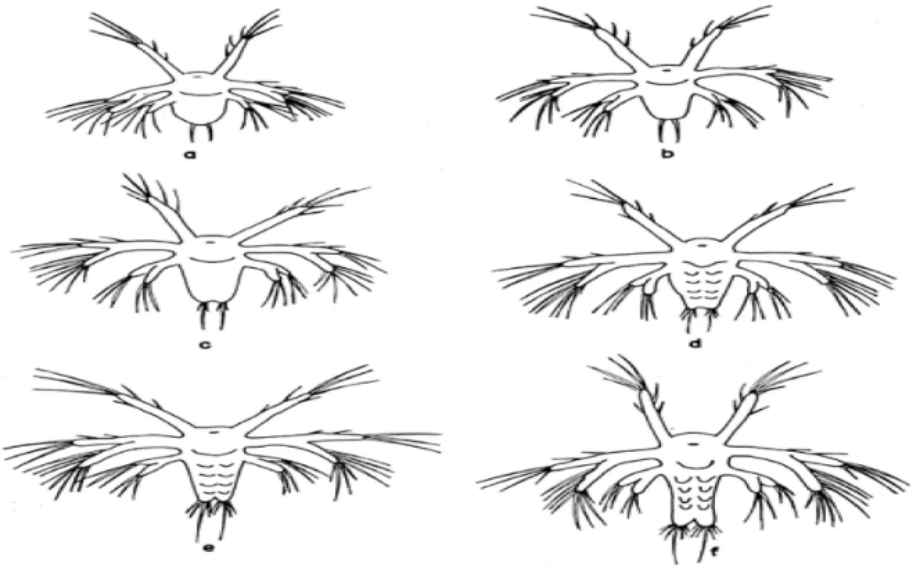
DESCRIPTION OF DEVELOPMENTAL STAGES EGGS

- The eggs are small with a narrow peri-vitelline space.
- The egg diameter varied from 0.25 to 0.27 mm and the yolk mass 0.22 to 0.24 mm.
- The radiating jelly like substance seen in the case of *P. monodon* is also present in the freshly laid eggs of this species.
- The developing nauplius almost fills up the entire space inside the egg.
- The eggs hatched out 12-15 hours after spawning.

Nauplius

- Nauplius stages are 6 from N1 to N6,
- Distal inner lateral seta long,
- Endopod with 4 setae, 2 inner lateral, 2 long terminal, a setal rudiment present terminally,
- Duration of this sub-stage was 3-4 hours.

a. Nauplius I b. Nauplius II c. Nauplius III
d. Nauplius IV e. Nauplius V f. Nauplius VI

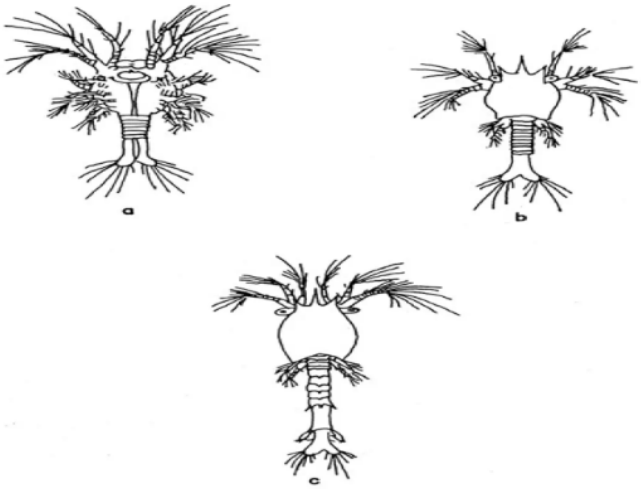


NAUPLIUS STAGES

PROTOZOEA

- Protozoea stages are 3 from Z1 to Z3,
- 6 setae on proximal endite and 4 setae on distal endite,
- Endopod with 3, 1, 2 and 5 setae,
- Duration of this substage was 36-48 hours.

a. Protozoea 1
b. Protozoea 2
c. Protozoea 3

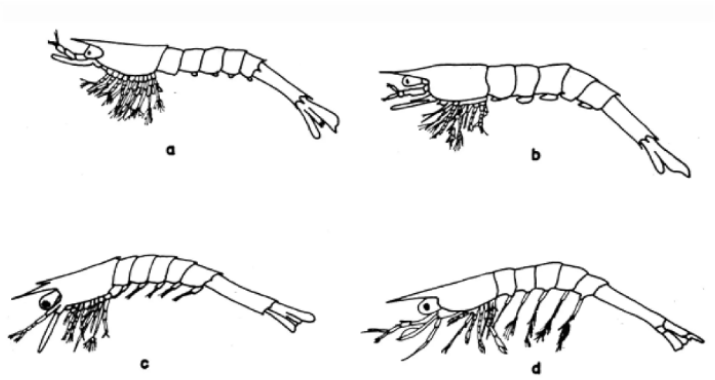


PROTOZOEAL STAGES

Mysis

- Mysis stages are 3 from M1 to M3,
- Exopod with 12 setae,
- 17-18 setae on exopod and 16 setae on endopod,
- Duration of this substage was 12-48 hours.

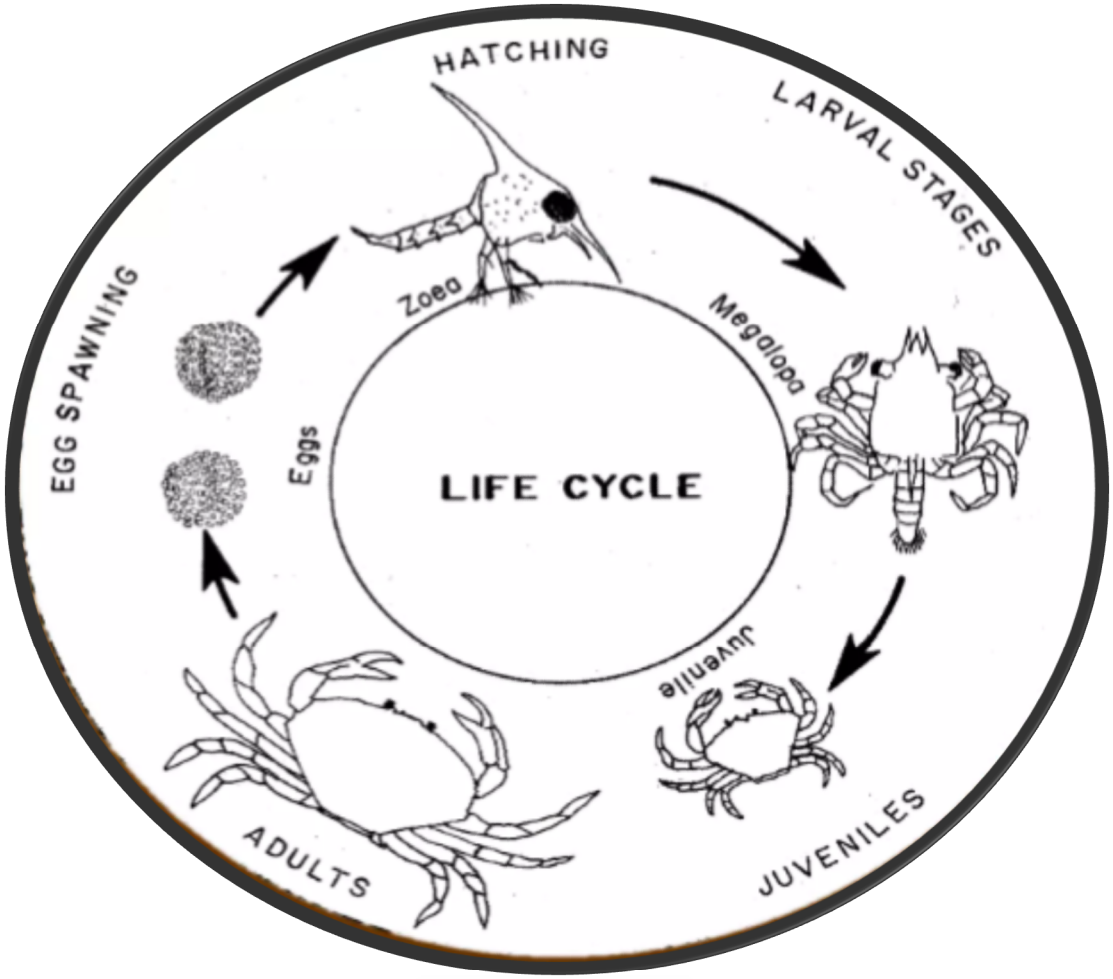
a. MYSIS 1 b. MYSIS 2 c. MYSIS 3 d. POSTLARVAE 1



Post Larve

- Post larve stages are 21 from PL1 to PL21,
- Endopod with 25 and exopod with 23 setae,
- Duration of this substage was 24 hours.

- Developmental stages of Crab.



Identification of larval stages of crabs (*Scylla serrata*)

Larval stages	Important morphological characters
Zoea I	Eyes sessile ; 5 abdominal segments; telson with 3 + 3 spines.
Zoea II	Eyes stalked ; 5 abdominal segments; telson with 4 + 4 spines.
Zoea III	6 abdominal segments
Zoea IV	Rudiments of remaining thoracic appendages, abdominal segments with buds of pleopod.
Zoea V	Remaining thoracic appendages developed; pleopods on abdominal segments with setae; telson with 5 + 5 spines.
Megalopa	Carapace longer than wider; abdomen with 5 pairs of pleopods ; a pair of cheliped; 4 pairs of legs.
First crab instar	Carapace with 9 anterolateral teeth on either side; 3 pairs of walking legs; last pair of legs with paddle shaped dactylus; resembles parent.

Larval stages of Mud crab

First Zoea

Eyes sessile
5 abdominal segments

Telson with
3+3 spines

Second Zoea

Eyes stalked
5 abdominal segments

Telson with
4+4 spines

Larval stages of Mud crab

Third Zoea

Abdominal
Segments

Fourth Zoea

Rudiments of remaing
Throracic Appendages,
Abdominal segments
with buds of pleopods

Fifth Zoea

Pleopodswith setae
Telson with
5+5 spines

Larval stages of Mud crab

Megalopa

First Crab Instar

Course No: ZOO 396B: Ecology Practical-I & Field trip

Preparation of Climograph

Purpose

To learn how to construct, read, and analyze climographs and understand how climate differs from weather.

Overview

Students calculate and graph monthly maximum and minimum temperature averages and monthly precipitation totals for a one-year period, then compare the data to a 30-year climograph (1981-2010) for the same approximate area. Students gain an understanding as to why at least 30-years of weather data are necessary to describe the climate for a given geographic location.

Background

Weather refers to the day-to-day atmospheric conditions in a given area over a short period of time from seconds to weeks. The condition of the atmosphere (hot, cold, sunny, windy…) refers to weather. Climate is the weather of a place averaged over a longer period of time (typically over 30 years). Climate refers to statistical averages of temperature, precipitation and other aspects of the atmosphere.

Climographs (also called climatographs) are a graphic way of describing the climate of a location including how temperature and precipitation vary through the year. Climatologists require a minimum of 30 years of temperature and precipitation data to adequately capture a location's "typical" climate.

A graph of one year of temperature and precipitation data can be helpful for understanding the weather that took place within a given year; however, it can fail to capture climate patterns for a location. In this activity, students explore the distinction between one year's weather data and the average of 30 years of weather data, learning that long-term measurements are important for describing the "normal" climate patterns of a place.

Comparing their climograph of one year of data with the 30-year climograph (see examples of each below), students will see how averaging data over many years can dampen the impact of unusual events such as usually dry months or heat waves. They should also notice that missing data makes the precipitation graph less accurate since it is a total for each month rather than an average.

What to Do and How to Do It

Step 1: The lesson will begin by asking students the difference between weather and climate. The following examples are added:

- *Climate is what we expect. Weather is what we get.*
- *Weather determines what you wear. Climate determines the clothes you own.*

Step 2: Hand out Reading: Climate and Weather. The difference between weather and climate are read and discussed as a class. In this activity it is learned how to make, read, and

analyze a climograph for one-year for a given location, then compare it to a 30-year climograph.

Step 3: Student Worksheet A and calculators are taken.

Step 4: An annual graph of temperature and precipitation data for _____ for the year _____ are created using the colored pencils, and the instructions provided on Student Worksheet A.

Step 5: The annual graph are compared with others in their group to ensure accuracy and completeness. Aclimograph usually includes 30 years of data.

Step 7: Students summarize what they learned.

Make a Climograph
Student Worksheet A

Name_____
Date_____

Details of location

_____
_____
_____
_____
___

Annual precipitation totals are calculated and mean monthly maximum and minimum temperatures using the _____ Data handout. Then a one-year climograph for _____ is made.

Part A: Monthly Precipitation Totals: Each month's precipitation totals in millimeters (mm) for each month are added. The calculated values in the table below are recorded.

Part B: Monthly Maximum Temperatures: The daily maximum temperatures for each day of the month are added, and the sum is dividedby the number of daily measurements made. The calculated values in the table below are recorded.

Part C: Monthly Minimum Temperatures: The daily minimum temperatures for each day within the month are added, and the sum is dividedby the number of daily measurements made. Record your calculated values in the table below.

Month	Monthly Precipitation Totals	Monthly Maximum Temperatures	Monthly Minimum Temperatures
January			
February			
March			
April			
May			
June			
July			
August			
September			
October			
November			
December			

Part D: One-year climatograph.

Three colored pencils (one light color and two darker colors), a ruler, and the graph template below (or spreadsheet software) are needed.

1. A bar graph is made of the monthly precipitation totals that are calculated in Part A on the graph below. Each bar is made the width of the dotted lines and colored with a light colored pencil. The precipitation is colored square in the key with the color used for the bar graph.

2. A darker colored pencil is chosen and a dot on the graph is made to represent the average maximum temperature for each month as calculated in Part B. A ruler is used to make sure each dot is in line with the name of the month. The dots are connected. The key for average high temperature is colored with the colored pencil.

3. With a different color of pencil, a dot is made for each of the average minimum temperature for each month using the values calculated in Part C. The dots are connected. The key for average low temperature is colored with the colored pencil.

Estimation of transparency, TSS, TDS, conductivity, hardness, salinity and alkalinity of water

Estimation of transparency of water by Secchi disc method

Principle

Turbidity in the water reduces light penetration. Light penetration in fact, depends partly on the light flux and mainly on the optical properties of water.

Light penetration in a body of water can be obtained by immersing secchi disc, which is a circular disc of metal of 20 cm in diameter painted matt white. Sometimes, a disc painted alternatively black and white in a radial fashion is also used. It has got a weight at the lower face so as to avoid a drift during lowering in water. A string is attached to it for lowering which may be marked in centimeters.

In conditions of bright light and calm water irradiance at the depth of secchi disc penetration is about 15% to that of just below the surface. The compensation level (photosynthesis and respiration) or the euphotic limit at which irradiance is 1% to that of the surface is nearly 2.5 times secchi disc depth. The vertical alternation co-efficient is about 1.9 times secchi disc depth.

Procedure

1. Lower the secchi disc in the water with the help of the string tied to it, until it just disappears. Note the depth by marking on the string.

2. Now uplift the secchi disc and note the depth at which it reappears again.

3. Repeat the procedure two times more at different points of the same pond.

4. For better result, measurement should be made during the middle of a sunny day.

Calculation

$$\text{Secchi disc penetration} = \frac{\text{point of disappearance + point of reappearance}}{2}$$

Experiment on Determination of Total Dissolved and Suspended Solids in Water

Aim

The aim of the experiments is determination of total, suspended and dissolved solids in water.

Apparatus Required
1. Balance
2. Beaker
3. Measuring Cylinder
4. Filter paper/ or Gooch Crucible
5. Funnel
6. Dropper

Procedure:

(a)Measurement of Total Solids (TS)
(1) Take a clear dry glass beaker of 150 ML capacity (which was kept at 103°C in an oven for 1 hour) and put appropriate identification mark on it. Weight the beaker and note the weight.

(2) Pour 100ml. of the thoroughly mixed sample, measured by the measuring cylinder, in the beaker.

(3) Place the beaker in an oven maintained at 103°C for 24hours. After 24 hours, when whole of the water has evaporated, cool the beaker and weight. Find out the weight of solids in the beaker by subtracting the weight of the clean beaker determined in step (1)

(4) Calculate total solids (TS) as follows:
Total Solids in water= Difference of weight of the beakers / Volume of sample X1000

(b) Measurement of Total Dissolved Solids (TDS)
(1) Same as above (step 1 of total solids).
(2) Take a 100 ml. of sample and filter it through a double layered filter paper or a Gooch Crucible and collect the filtrate in a beaker.
(3) Then repeat the same procedure as in steps (3) and (4) of the total solids determination and determine the dissolved solids contents as follows:

Calculation:
Dissolved solids, TDS (mg/l) = mg of solids in the beaker / (volume of sample) x 1000

Also total solid (TS) = Suspended Solids + Total dissolved Solids (TDS)

Estimation of alkalinity of water

Introduction:

The alkalinity of the water is a measure of its capacity to neutralize acids. The alkalinity of natural waters is due primarily to the salts of week acids. Bicarbonates represent the major form of alkalinity. Alkalinity can be expressed as follows: Alkalinity (mol/L) = $[HCO_3^-] + 2[CO_3^{2-}] + [OH^-] - [H^+]$

Alkalinity is significant in many uses and treatments of natural waters and wastewaters. As alkalinity of many surface waters constitute of carbonates, bicarbonate and hydroxide contents, it is assumed to be an indicator of these constituents as well. Alkalinity in excess of alkaline earth metal concentrations is significant in determining the suitability of

water for irrigation. Alkalinity measurements are used in the interpretation and control of water and wastewater treatment processes. Raw domestic wastewater has an alkalinity less than or only slightly greater than that of the water supply.

Requirements:
Apparatus: Burette, conical flask, pipette, measuring cylinder
Reagents: H_2SO_4 solution, Phenolpthalein indicator, Methyl Orange indicator

Procedure:
1. Fill the burette to H_2SO_4 solution.

2. Take a 100ml water sample in flask. Add few drop of Phenolphthalein indicator.

3. Note the initial reading on burette scale. Titrate against H_2SO_4 till the pink colour disappears.

4. Note the end point reading and get volume of used H_2SO_4 in ml (P) (Concordant value I).

5. Add 1-3 drop of Methyl Orange in same sample flask.

6. Titrate it, till the appearance of light orange colour.

7. Note down the final reading and find the volume of used H_2SO_4.

8. Repeat the steps of using the sample to get concordant value (Concordant value II).

9. Calculate the total alkalinity of sample.

Observation:
Observation table:
For Concordant value I: Determination of phenolphthalein end point

S.No.	Water sample	Initial Value	Final Value	Ml of H_2SO_4 (P)
1				
2				
3				

For Concordant value II: Determination of methyl orange end point

S.No.	Water sample	Initial Value	Final Value	Ml of H_2SO_4
1				
2				
3				

Calculations:
Total volume of standard H_2SO_4 used for the titration:
T = Concordant value I + Concordant value II

Thus, Phenolpthalein alkalinity = P*1000/ml sample
Total alkalinity (mg/lit of CaCO3) = T*1000/ml sample

Estimation of Salinity of Water Sample

Introduction

Salinity refers to the total amount of soluble salts dissolved in a kilogram of water collectively. The salts in water include such common ions as Ca^{2+}, Mg^{2+},K^{+},Na', $C1^{-}$, SO_2^{-}, HCO_3^{-} etc. These ions occur either naturally or added as pollutants to the environment. The ionic composition of water affects the distribution of animals and plants in water.

Materials Required
1. 100 ml conical flasks- 2
2. 10 ml pipettes- 2
3. 50 ml burette
4. 0.05 N Silver nitrate solution ($AgNO_3$)
5. 5% Potassium chromate solution
6. Water samples- 2 different water samples (A and B)

Procedure
1. The burette was filled with 0.01 N $AgNO_3$, solution.

2. 10 ml of water sample A was taken in a conical flask and a few drops of 5% Potassium chromate solution was added.

3. The water samples were titrated against $AgNO_3$, solution. The end point was the appearance of brick red colour.

4. The sample was titrated until the concordant values were obtained. Titration was done a minimum of two times.

5. The results were recorded in the form of following table in record note book.

6. The experiment was repeated with sample B.

Sl. No.	Volume of sample	Burette reading		Volume of AgNO3 consumed
		Initial	Final	

Calculations

Chlorosity of Water =

$$\frac{\text{Volume of } \mathbf{AgNO_3} \text{ consumed } \mathbf{x} \text{ Normality of } \mathbf{AgNO_3}}{\text{Volume of the sample.}}$$

Chlorinity of Water = $\frac{\text{Chlorosity of water}}{\text{density of water}}$

For practical purposes, the density of water can be takes as 1.
Salinity of Water = 0.03 + (1.805 **x chlorinity** of water)

= ..... parts per thousand

ESTIMATION OF PHOSPHORUS IN SOIL

SCOPE

This test outlines the procedure for the determination of available phosphorus in soils. Bray No 1 solution is designed to extract adsorbed forms of phosphate only and is for use with soils with a pH <7.5.

PRINCIPLE

Phosphorus is extracted from the soil using Bray No 1 solution as extractant. The extracted phosphorus is measured colourimetrically based on the reaction with ammonium molybdate and development of the 'Molybdenum Blue' colour. The absorbance of the compound is measured at 882 nm in a spectrophotometer and is directly proportional to the amount of phosphorus extracted from the soil.

SPECIAL APPARATUS

• Centrifuge (6 000 rpm).

• Diluter/Dispenser (Brand Diluette® Cat No 7046 54).

• High strength centrifuge tubes with caps (15 mL capacity).

• Spectronic 20 photometer with 10 mL tubes.

Reagents

Bray No 1 Extracting Solution

Dissolve 2.22 g Ammonium Fluoride A.R. (NH4F) in deionised water and transfer to a 2 L volumetric flask. Add 5 mL concentrated hydrochloric acid and bulk to volume with deionised water.

Reagent A

Dissolve 17.14g Ammonium molybdate A.R. $[(NH4)_6Mo_7O_{24}.4H_2O]$ in 200 mL of warm deionised water. Dissolve 0.392 g potassium antimonyl tartrate A.R. $(KSbO.C_4H_4O_6)$ separately in 150 mL deionised water. Place 500 mL deionised water in a 2 L volumetric flask and slowly add 200 mL concentrated sulphuric acid with mixing. When cooled, add the cooled molybdate and tartrate solutions, then mix and bulk to volume with deionised water.

Reagent C

Dissolve 0.53 g L-Ascorbic Acid A.R. $(C_6H_8O_6)$ in deionised water and transfer to a 500 mL

volumetric flask. Add 70 mL of Reagent A and bulk to volume with deionised water. Prepare fresh a volume of this solution sufficient for the day's work by proportioning the above quantities.

Standard Phosphorus Solution (P ≡ 50 mg/L)

Dissolve 0.2195 g potassium dihydrogen orthophosphate A.R. (KH_2PO_4) in 100 mL deionised water, transfer to a 1 L volumetric flask, add 5 mL concentrated sulphuric acid (A.R.) and bulk to volume with deionised water.

Phosphorus Working Standard (P ≡ 2.50 mg/L)

Pipette 5 mL standard phosphorus solution into a 100 mL volumetric flask and bulk to volume with deionised water.

Procedure

1. Dispense 7 mL Bray Extracting Solution into the oven-dry equivalent of 1 g of air-dry soil contained in a centrifuge tube. Include one tube containing the Bray Solution only for the blank.

2. Stopper the tube and shake vigorously for 1 minute.

3. Transfer the tubes to the centrifuge and spin at 6 000 rpm for 5 minutes.

4. Dispense 0.50 mL of the supernatant plus 2.0 mL Reagent C into a colourimeter tube. Mix and stand for 30 minutes.

5. Prepare a set of reference standards from the 2.50 mg/L phosphorus solution using the diluter/dispenser and the following table as a guide:

Table 1

DILUTION TABLE FOR STANDARDS			
mls 2.5mg/L Ref.	mls Reagent C	Phos. Conc. (µg/2.5 mL)	Typical Absorbance Value
0.05	2.45	0.125	0.06
0.10	2.40	0.250	0.09
0.20	2.30	0.500	0.17
0.30	2.20	0.750	0.26
0.40	2.10	1.000	0.34
0.50	2.00	1.250	0.40

6. Set instrument zero (∞ Abs.) and then set full scale (zero Abs.) using the blank solution prepared above.

7. Measure and record the absorbance of the standards and samples at wavelength 882 nm.

8. Prepare a chart or graph from the standards data to plot phosphorus concentration against absorbance, or derive the equation of the line of best fit using linear regression. Use the chart or equation to determine the phosphorus concentration in the sample solutions.

Calculations

Calculate Available Phosphorus content.

$$Available\ Phosphorus\ (mg/kg) = \frac{C\ x\ 14}{ODW}$$

Where: C = Phosphorus concentration from chart/equation (µg/2.5 mL)

ODW = Oven-dry sample weight (g)

14 = Dilution factor

Collection, preservation, identification and analysis of aquatic biota and Ecological comments on major biotic components in Aquatic system

Introduction

The term *plankton* was first used by German biologist Victor Hensen in 1887. According to him plankton includes all organic particles which drift freely and involuntarily in open water, independent of shores and bottom. They have either relatively small power of locomotion or not at all. Plankton differs greatly in size and species.

Classification

Plankton can be broadly classified into 2 groups-

1. **Phytoplankton**- Theyincludes plants. Most of the phytoplankton survives on the open surface waters of lakes, rivers and oceans. Its community comprises of algal members from both prokaryotes and eukaryotes. It includes Cyanobacteria, Chlorophyta, Euglenophyta, Cryptophyta etc.

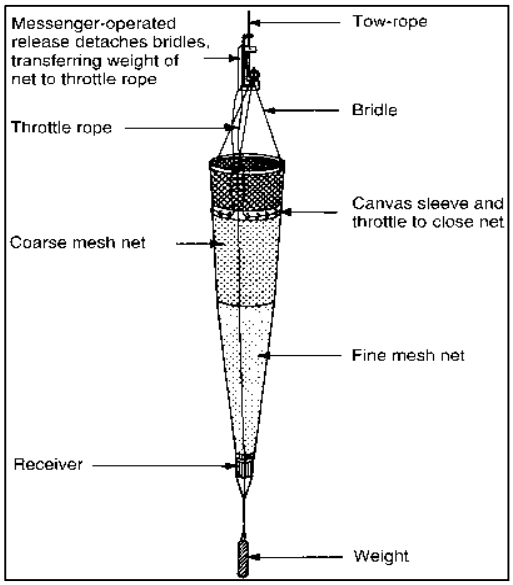
2. **Zooplankton**- They includes animals. Zooplanktonic population of a fresh water biota may be composed of Arthropods like cladocerans, copepods and ostracods along with pseudocoelomates like rotifers.

Plankton Net

Plankton nets are the most popularly used device for sampling and may vary in design that range from basic to tow nets to more complicated device fitted apparatus for more specific sample collection. Nets permit quantitative studies, since the mesh size will select the type of plankton collected. Sampling by nets is highly selective, depending on mesh size of the gauze, net towing speed and the species present in the water.

Structure of plankton net-A typical plankton net usable in the surface layers is conical in shape and has the following constituents:

- A net ring made up of stainless steel, wrapped and sealed with a polythene tude, present anteriorly.
- A non-filtering portion made of coarse khaki cloth is attached using button and hole system.
- The filtering portion is made of monofilament nylon material.



The determination of the volume of water filtered through any plankton net is essential for the estimation.

Materials required

Plankton net, 70% ethanol, plastic bucket, Sample bottle, Microscope, slide, cover slip, dropper and Sedgwick-Rafter Cell.

Procedure

a. *Collection and Preservation of planktons*

1. Planktons can be collected by filtering a known quantity of water through plankton net No. 25, mesh size 55µm. The surface water for this purpose may be collected with the aid of a plastic bucket of specific volume.

2. Plankton net weighted at the base is lowered into the water to known depth with the help of a graduated rope. The net is then pulled gradually out. The volume of water filtered through the plankton net is calculated

$$\mathbf{V}=\pi r^2$$

Where, V= volume of water (L)
π= 3.142
r= radius of mouth of plankton net (m)
h= depth up to which the net was lowered

Plankton collected is transferred to a small sample bottle (50-100 ml) and preserved in 70% ethanol.

b. *Quantitative analysis of plankton population* (**Sedgewick Rafter Method**)
1. Concentrated sample is shaken well and without delay, 1 ml of it is transferred in the cavity of Sedgewick Rafter cell with a dropper.

2. Cavity of the cell is covered with a cover slip avoiding any air bubble to get in. planktons are allowed to settle and then counted under a microscope.
3. At least 10 replicates are taken and the average count per ml is calculated.
Calculation

$$\textbf{Zooplankton (Units/L)} = \frac{\mathbf{N} \times \mathbf{C}}{\textbf{Volume of sample in L}}$$

Where, **N**= number of plankton counted in 1 ml concentrate
C= total volume of concentrate (ml)
Volume of sample in L= total volume of water taken

c. *Qualitative analysis of plankton population*

Each type of plankton is separated by sieving and then microscopic observation of the planktons is done for Genus and species identification. Separated planktons are preserved in 70% alcohol.

Identification-
Pelagic Community-
Zoolplankton
1. ***Daphnia* sp.**
Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Branchiopoda

Order-Cladocera
Type-*Daphnia*

Distinctive features
1. Bilaterally compressed body, enclosed in a transparent bivalve carapace, ending into sharp caudal spine and anteriorly into pointed rostrum.
2. Rounded head bears large **biramous antennae**.
3. Large, sessile, single, huge eye is very distinct.
4. Five pairs of leaf like thoracic appendages and abdomen devoid of appendages.
5. In females, there is a brood pouch near the back.

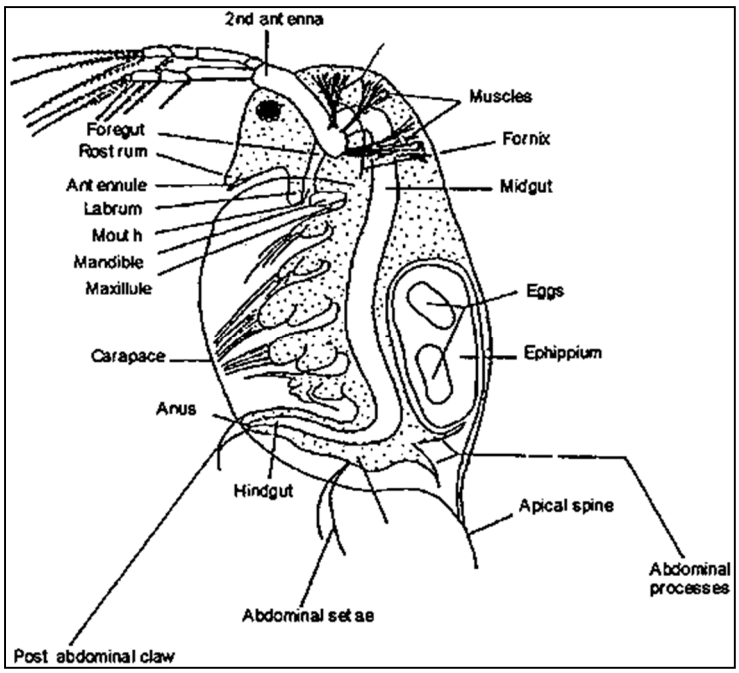


Fig 1. *Daphnia* sp.

Ecological comments

Daphnia often plays the role of keystone species in ponds and lakes, where they are the most important primary consumer, filter-feeding small suspended particles, particularly algae. They also play an important role in aquatic food webs by being the food reserve for fish and other invertebrate predators.

2. ***Moina* sp.**
Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Branchiopoda
Order-Anomopoda
Type-*Moina*

Distinctive features
1. Body oval shaped.
2. Antennae are branched.
3. Rostrum is in horizontal arrangement.
4. Body terminates into a pair of caudal styles.

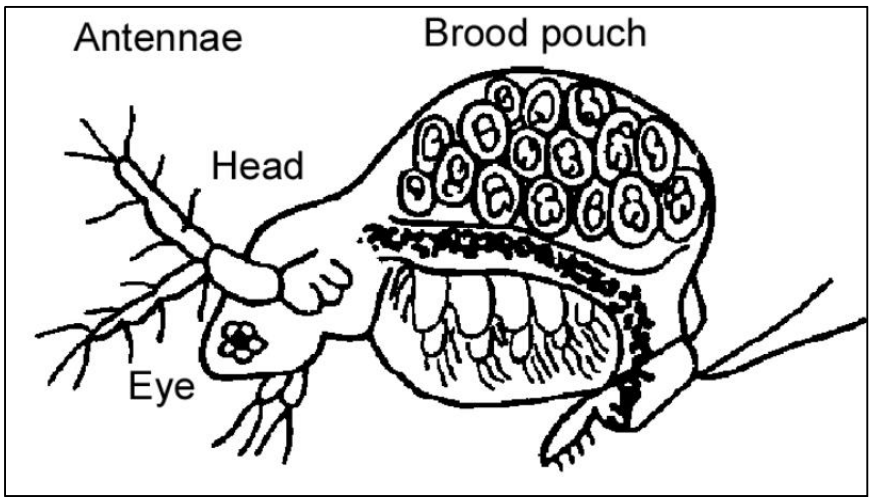


Fig 2. *Moina* sp.

Ecological comments

Moina feed on various groups of bacteria, yeast, phytoplankton and detritus and they are live food for finfish, crustaceans, teleost and marine fishes because of their high protein and nutrient content thus maintaining the aquatic food web. They are often used a feed in aquaculture thus having a high economic value.

3. ***Cyclops* sp.**

Systematic Position

Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Copepoda
Order-Cyclopoida
Type-*Cyclops*

Distinctive features

1. Pear shaped body with broad anterior and narrow posterior end.
2. The head and first thoracic segment fuse to form cephalothorax.
3. A median eye is present near the anterior end.

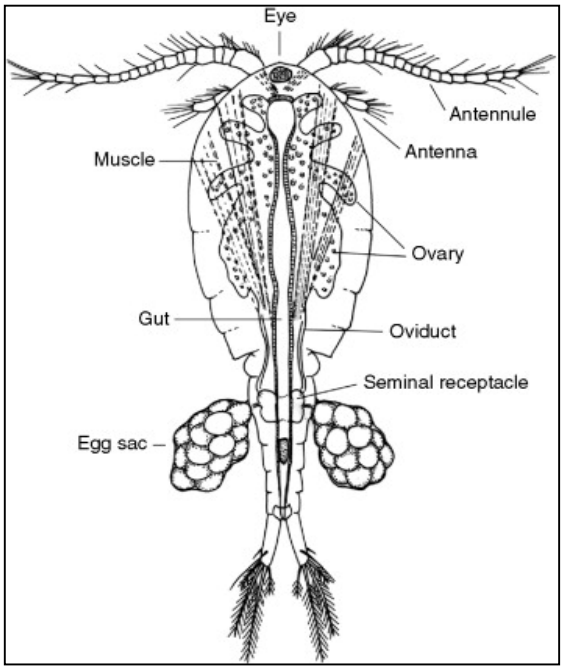


Fig 3. *Cyclops* sp.

Ecological comments

Cyclops play a bridging role in the aquatic food web by consuming dinoflagellates and phytoplanktons thus providing food sources for higher level predatory nekton species. It is the intermediate host of guinea-worm disease and fish tapeworm infection and can be passed to humans through infected water consumption.

4. ***Cypris* sp.**

Systematic Position

Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Subclass-Ostracoda
Type-*Cypris*

Distinctive features

1. The body is entirely enclosed within a bivalve carapace.
2. Well developed antennae used in swimming.
3. Gills enclosed within the valves.
4. Head bears four pairs of appendages.
5. Presence of three pairs of thoracic appendages.

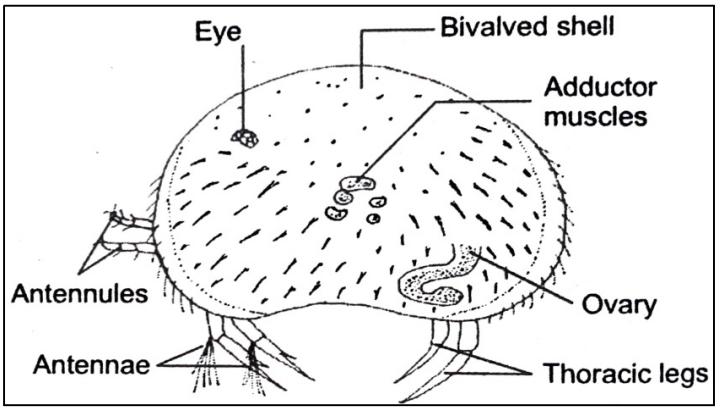


Fig 4. *Cypris* sp.

Ecological comments

Cypris has an important role in the aquatic ecosystem serving as vital food sources for larger aquatic predators like fish, other crustaceans, benthic molluscs etc. They can also recycle nutrients as filter feeders

5. ***Nauplius* larvae**

Systematic Position

Phylum- Arthropoda
Subphylum-Mandibulata
Class-Crustacea
Type-*Nauplius*

Distinctive features

1. Minute, conical and microscopic with broad anterior and narrow posterior end.
2. Body is oval and divided into indistinct head, trunk and anal region.
3. It contains single median eye and frontal and lateral horns.
4. Three pairs of appendages namely uniramous antennules, biramous antennae and biramous mandibles.
5. Larvae contain mouth and gut.

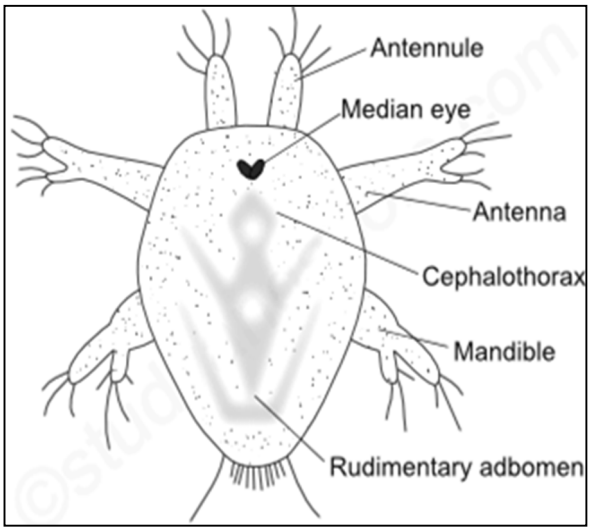


Fig 5. Nauplius larva of *Cyclops* sp.

Ecological comments

The Naplius larvae sometimes functions as food gatherers and has a well-developed alimentary system storing the food so that the transformation to the adult stage can occur.

6. ***Volvox* sp. (Phytoplankton/Periphyton)**

Systematic Position

Division- Chlorophyta

Class-Chlorophyceae

Distinctive features

1. Free floating fresh water green algae.
2. Each mature *Volvox* colony is composed of up to thousands of cells from two differentiated cell types.
3. Adult somatic cells comprise a single layer with the flagella facing outward.
4. The cells have anterior eyespots that enable the colony to swim toward light.
5. *Volvox* is facultatively sexual and can reproduce both sexually and asexually.

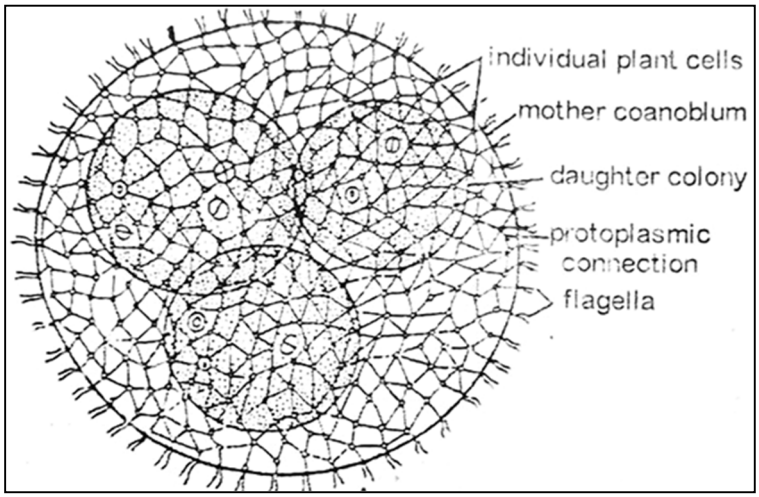


Fig 6.*Volvox* sp.

Ecological comments

They can be found in ponds and other fresh water bodies throughout the world. They contribute to the production of oxygen and serve as food for a number of aquatic organisms like rotifers.

Benthic Community-

1. ***Ephemera* sp.** (May flylarvae)

Systematic Position

Phylum- Arthropoda

Subphylum-Mandibulata

Class-Insecta

Subclass- Pterygota

Order-Ephemeroptera

Type-*Ephemera*

Distinctive features

1. Sides of the abdomen with plate like, feather like or leaf like gills, usually with three terminal filaments.
2. Tarsal claw single.
3. Older larvae show developing wing pads.
4. Gills feathery, consists of two branchiaes each thickly fringed with filaments.
5. Gills held over back during life.
6. Mandibles project beyond front of the head.

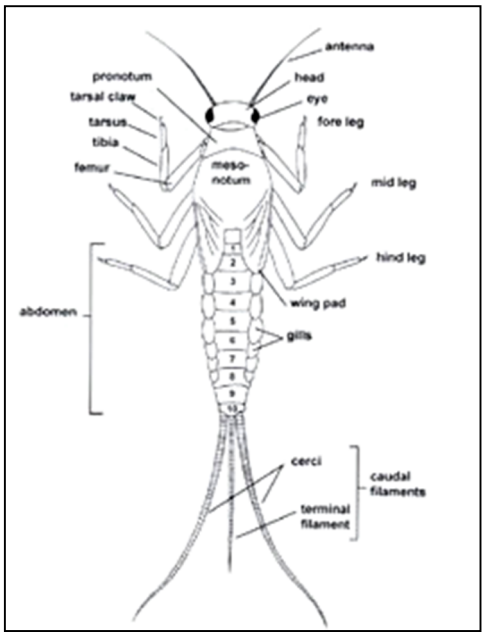


Fig 7. *Ephemera* sp. (May fly larvae)

Ecological comments

Mayflies are especially important to fishing contributing to the provisioning services of ecosystems in that they are utilized as food by human cultures worldwide, as laboratory and as potential source of anti tumour molecules.

2. **Dragon fly nymph**

Systematic Position

Phylum- Arthropoda
Subphylum-Mandibulata
Class-Insecta
Subclass- Pterygota
Order-Odonata
Type-Dragon fly nymph

Distinctive features

1. Labium prominent, extensible forming a food capturing structure larger than head.
2. Abdomen terminating in three to five stiff pointed valves, longest less than one third of abdomen.

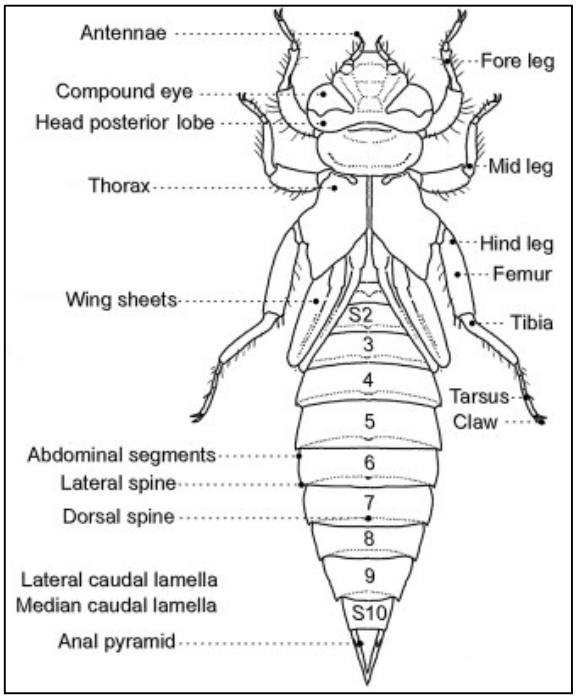


Fig 8. Dragon fly nymph

Ecological comments

They are freshwater insects showing semi aquatic life cycle. They also play an important role in controlling other disease spreading insect population such as mosquitoes acting as underwater predators.

Aquatic insect-

1. ***Belostoma* sp.** (Giant water bug)

Systematic Position
Phylum- Arthropoda
Subphylum-Mandibulata
Class-Insecta
Subclass- Pterygota
Order-Hemiptera
Type-*Belostoma*

Distinctive features
1. Large insect with elongated body covered by leathery brownish exoskeleton.
2. Mouth parts piercing and sucking type.
3. Legs flat, forelegs shorts and raptorial while posterior legs adopted for swimming and crawling.
4. Thorax broad and somewhat triangular.

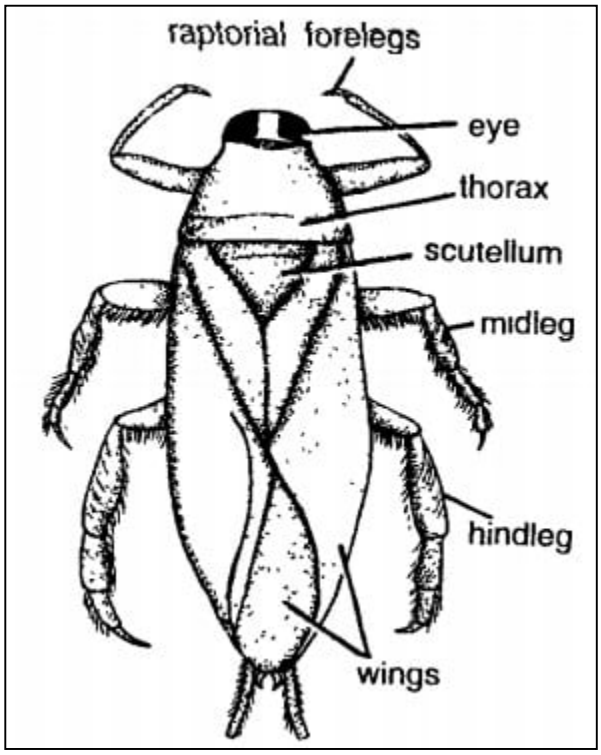


Fig 9. *Belostoma* sp.

Ecological comments

Belostoma inhabit permanent lentic habitats, especially weedy ponds, margins of lakes and marshes. They are important as apex invertebrate predators.

Macrophytes

1. ***Pontederia crassipes*** (Common water hyacinth)

Systematic Position
Kingdom- Plantae
Order-Commelinales
Family-Pontederiaceae
Type-*Pontederia crassipes*

Distinctive features
1. Floating waterweed up to 65 cm tall and the leaves are dark green rounded leaves up to 5–10 cm in diameter.
2. Leaf stalks of young plants are swollen into spongy, bulbous structures; mature plants have elongated leaf stalks.
3. An erect stalk supports a single spike of 8 to 15 flowers. Flowers are light purple with a darker blue-purple with yellow centres.
4. The root system is extensive feathery, fibrous, black to purple.

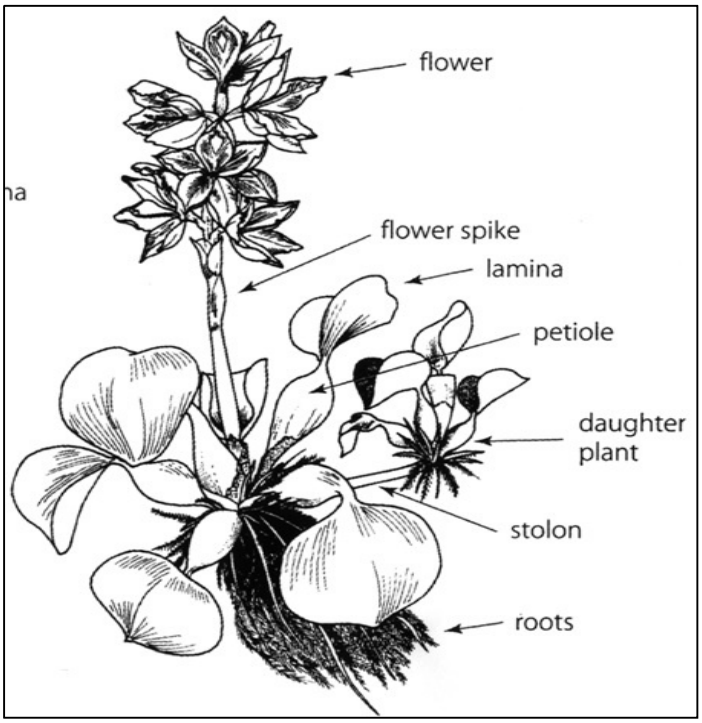


Fig 10. Common water hyacinth

Ecological comments
It has immense potential for use as an energy resource. It is used as an energy feedstock with its potential capacity for phytoremediation due to its dense population and aggressive growth.

2. ***Spirodela polyrhiza*** (Duck weed)

Systematic Position
Kingdom- Plantae
Order-Alismatales
Family-Araceae
Type-*Spirodela polyrhiza*

Distinctive features
1. Perennial aquatic plant usually growing in dense colonies forming a mat on water surfaces.
2. Each plant is a smooth, round, flat disc 0.5 to 1.0 cm wide with mostly green or red upper surface while lower surface is dark red.
3. Produces several minute roots and a pouch containing male and female flowers.

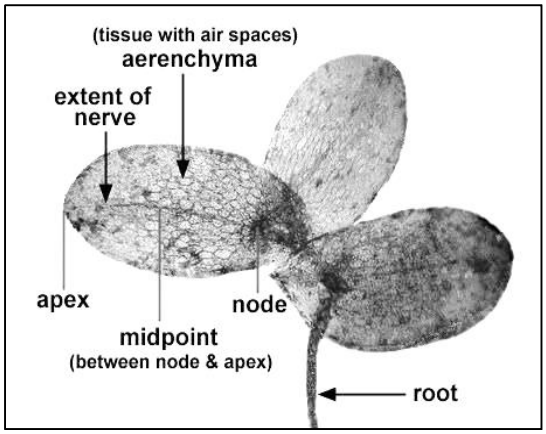


Fig 11. Duckweed

Ecological comments

Phytoremediation using duckweeds is a cost-effective and environment friendly strategy to prevent environmental pollution and preserve aquatic and terrestrial ecosystems although proper disposal of contaminated duckweeds should be considered.

Applicability of GPS in recording bioresources and mapping the landscape.

Up until now, we've looked at how you can use GPS receivers to tell you where you are, to navigate between points and to make digital maps of various features. But GPS isn't just used by civilians; it's also used by pilots, boat captains, farmers, surveyors, scientists and the military (just to name a few!).

While typical civilian handheld GPS receivers are usually accurate to about 5 metres, there are also very expensive, highly advanced GPS receivers that are capable of providing positions accurate to within a centimeter. These receivers have revolutionized lots of industries, where highly accurate positioning is used for so many different tasks. The following sections provide a quick summary of how GPS is used in some industries.

Aviation

Almost all modern aircraft are fitted with multiple GPS receivers. This provides pilots (and sometimes passengers) with a real-time aircraft position and map of each flight's

progress. GPS also allows airline operators to pre-select the safest, fastest and most fuel-efficient routes to each destination, and ensure that each route is followed as closely as possible when the flight is underway.

Marine

When high accuracy GPS is fitted to boats and ships, it allows captains to navigate through unfamiliar harbours, shipping channels and waterways without running aground or hitting known obstacles. GPS is also used to position and map dredging operations in rivers, wharfs and sandbars, so other boats know precisely where it is deep enough for them to operate.

Farming

Farmers rely on repeat planting season after season to maximise their crop productions. By putting GPS receivers on tractors and other agricultural equipment, farmers can map their plantations and ensure that they return to exactly the same areas when sewing their seeds in future. This strategy also allows farmers to continue working in low-visibility conditions such as fog and darkness, as each piece of machinery is guided by its GPS position instead of visual references. High accuracy GPS is also used to map soil sample locations, allowing farmers to see where the soil is most fertile across individual fields or even entire farms.

Science

Scientists use GPS technology to conduct a wide range of experiments and research, ranging from biology to physics to earth sciences. Traditionally, when scientists wanted to understand where and how far animals roam, they had to tag animals with metal or plastic bands and then follow them to various locations to monitor their movement. Today, scientists can fit animals with GPS collars or tags that automatically log the animal's movement and transmit the information via satellite back to the researchers. This provides them with more detailed information about the animal's movements without having to relocate specific animals.

Earth scientists also use GPS technology to conduct a wide range of research. By installing high accuracy GPS receivers on physical features such as glaciers or landslips, scientists can observe and study both the speed and direction of movement, helping them to understand how landscapes change over time. Similarly, GPS receivers can be installed on solid bedrock to help understand very small and very slow changes in tectonic plate motion across the world.

Surveying

Surveyors are responsible for mapping and measuring features on the earth's surface and under water with high accuracy. This includes things like determining land boundaries,

monitoring changes in the shape of structures or mapping the sea floor. Surveyors have historically required line-of-sight between their instruments in order to undertake such work, but the availability of high accuracy GPS receivers has reduced the need for this. GPS can either be setup over a single point to establish a reference marker, or it can be used in a moving configuration to map out the boundaries of various features. This data can then be transferred into mapping software to create very quick and detailed maps for customers.

Military

The GPS system was originally developed by the United States Department of Defence for use by the US military, but was later made available for public use. Since then, GPS navigation has been adopted by many different military forces around the world, including the Australian Defence Force. Some countries have even decided to develop their own satellite navigation networks for use during wartimes. Today, GPS is used to map the location of vehicles and other assets on various battlefields in real time, which helps to manage resources and protect soldiers on the ground. GPS technology is also fitted to military vehicles and other hardware such as missiles, providing them with tracking and guidance to various targets at all times of the day and in all weather conditions.

396C: Genetics & Molecular Biology Practical & Institute /Lab visit

Preparation of mitotic metaphase chromosome of Rat.

Genomic instabilities including chromosomal translocations are frequently associated with genetic diseases and cancer, especially leukemia. Cytogenetic studies of these diseases requiring preparation of metaphase chromosomes are often key to reveal their chromosomal abnormalities.

Chromosome preparations for cytogenetic analysis are made from dividing cells that is directly from tissue samples like bone marrow, testes, chorionic villi, neoplastic tissue. Tissue preserved in a fixative is not suitable for making conventional chromosome preparations.

Required materials

1. 0.04% colchicine: Dissolve 0.04 g colchicine in 100 ml sterile distilled H_20 and store at 4°C.
2. 0.67% KCl: (0.67 g in 100 ml deionized H2O; freshly prepared)
3. Fixative (3:1 methanol/ ethanol : glacial acetic acid; freshly prepared)
4. Giemsa stain
5. 1 cc disposable syringes with 23 gauge needles
6. Micropipet (1ml, 200µl, 20 µl and 3/4" Pasteur pipets)
7. 1.5 ml Microfuge
8. Cold Centrifuge

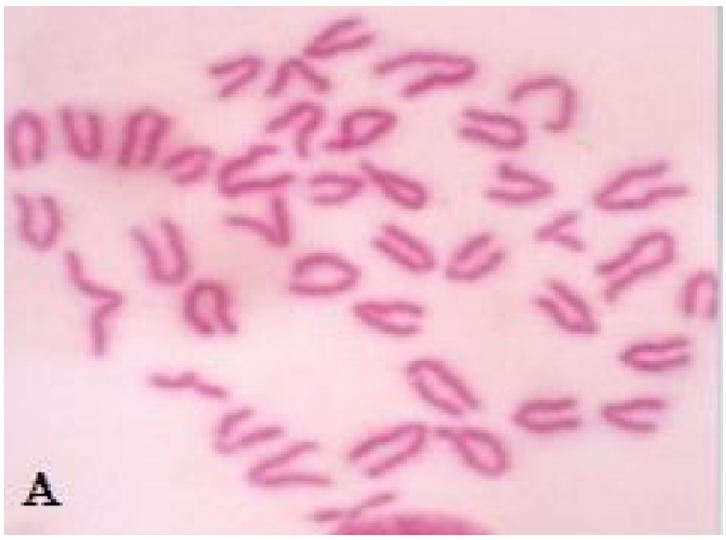
Bone marrow procedure

1. Inject 0.04% colchicine, 1 ml /100 gm body weight to the animal 2-3h prior to sacrifice
2. Sacrifice by cervical dislocation and dissect out femur bone (any long bone will do) Sacrifice rat and remove femur(s) and tibia(s). Early metaphases seem to be more prevalent in tibias.

3. Cut off just enough of the bone heads to insert a 23-gauge needle into the marrow cavity.
4. Flush out cells into a conical centrifuge tube using a 1 cc syringe filled with 0.67% KCl.
5. Incubate the tubes at room temperature (or 37° C if room is cool) for 15 min.
6. Centrifuge at 8000 rpm for 10 min. in a clinical bench-top centrifuge.
7. Remove supernatant and add 0.5 ml of fixative without disturbing the pellet. Remove fixative after 3-4 sec. and add 2 ml fresh fixative without disturbing the pellet.
8. Allow tubes to sit at room temperature 30 min. The procedure can be interrupted at this point and resumed later. Always refrigerate cells if they are to be left standing in fixative longer than 30 min.
9. After 30 min. centrifuge the cells at 8000rpm, remove the fixative, and resuspend the cells in fresh fixative.
10. Repeat step 9 twice more. The last addition of fixative should be just enough to make a thin cell suspension (solution in tube will look slightly opaque).

Chromosome slide preparation:

- Slides should be clean and grease free. Slides should be kept in cold ethanol overnight in refrigerator.
- Immerse pre-cleaned slides in fixative at least 15 min. prior to use. Wipe slides dry with a tissue paper.
- Drop small drops of cell suspension onto slide surface with a Pasteur pipet and allow it to spread. If too much suspension is used, it will bubble at the edges.
- As soon as the drop begins to contract and Newton's rings are visible (rainbow colors around the edge of the drop), blow on the slide surface to accelerate drying.
- After proper drying, stain the slide with Giemsa for 3-4min and following 2 rinses in distilled water (pH 6.8-7.2) or clean tap water, air dry the slide. When completely dried, mount with DPX mountant, using a large coverglass .
- To begin with, prepare only one slide and observe under the microscope to judge the density of cell suspension (an unstained slide can be examined for this purpose). If there are too many cells on the slide, dilute the suspension by adding more fixative. If the cells are too few, re-spin the tube and suspend the cells in a smaller volume of fixative.



Preparation of Genomic DNA from Bacteria

Bacteria from a saturated liquid culture are lysed and proteins removed by digestion with proteinase K. Cell wall debris, polysaccharides, and remaining proteins are removed byselective precipitation with CTAB, and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Required Reagents

i. TE buffer

ii. 10% sodium dodecyl sulfate (SDS)

iii. 20 mg/ml proteinase K (stored in small single-use aliquots at -20^0 C)

iv. CTAB/NaCl solution

v. 24:1 chloroform/isoamyl alcohol

vi. 25:24:1 phenol/chloroform/isoamyl alcohol Isopropanol

vii. 70% ethanol

viii. 5 M NaCl

Procedure

1. Inoculate a 5-ml liquid culture with the bacterial strain of interest. Grow in conditions appropriate for that strain (i.e., appropriate medium, drug selection, temperature) until the culture is saturated. This may take several hours to several days, depending on the growth rate.

2. Spin 1.5 ml of the culture in a microcentrifuge for 2 min, or until a compact pellet forms. Discard the supernatant.
3. Resuspend pellet in 567 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDSand 3 µl of 20 mg/ml proteinase K to give a final concentration of 100 mg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 1 hr at 37^0C.
4. Add 100 µl of 5 M NaCl and mix thoroughly.
5. Add 80 µl of CTAB/NaCl solution. Mix thoroughly and incubate 10 min at 65^0C.
6. Add an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol, mix thoroughly, and spin 4 to 5 min in a microcentrifuge.
7. Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge for 5 min.
8. Transfer the supernatant to a fresh tube. Add 0.6 vol isopropanol to precipitate the nucleic acids (there is no need to add salt since the NaCl concentration is already high). Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. At this point it is possible to transfer the pellet to a fresh tube containing 70% ethanol by hooking it onto the end of a micropipet that has been heat-sealed and bent in a Bunsen flame. Alternatively, the precipitate can be pelletedby spinning briefly at room temperature.
9. Wash the DNA with 70% ethanol to remove residual CTAB and respin 5 min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet in a lyophilizer.
10. Redissolve the pellet in 100 µl TE buffer.

Plasmid isolation and restriction digestion and Agarose gel electrophoresis

Principle:

Plasmid is an extra chromosomal circular self-replicating double stranded DNA molecule found in bacterial cell. For plasmid isolation bacterial culture should be grown to late logarithmic on stationary phase. This buffer is the typical buffering substance for DNA because it is slightly alkaline and DNA also be stored best pH 7.5-8.2. EDTA is an important substance in plasmid isolation because it inhibits nuclease activity.

Requirements:

1. Microfuse tubes
2. Micropipette and tips
3. Reagents:
    i. TE buffer- 1 M Tris buffer 0.5 M EDTA Solution
    ii. 10mM EDTA solution
    iii. Solution I (pH 8) : 50 M glucose + 10mM EDTA +25mM Tris buffer stored at 2-8 ^{0}C
    iv. Solution II (pH 7) : 0.2N NaOH +1% SDS stored at RT
    v. Solution III (pH 6) : 3M Potassium acetate & stored at 2-8 ^{0}C
    vi. DNA loading Dye: 0.05% Bromophenol blue& 10% glycerol.
    vii. Ethidium bromide; (EtBr); 10mg/ml
    viii. Running Buffer (pH-8) 48gm Tris +0.115gm Acetic acid + 0.5 M EDTA

Procedure:

i. At first LB media was Prepared and autoclaved after that 40µg/ml of antibody was added .
ii. 500 µl of sample (Plasmid containing Bacteria) is transferre into newly prepared LB media & allowed for incubation overnight at 37 ^{0}C in stable condition.

Isolation of Plasmid:

i. Firstly newly prepared bacterial culture was taken in eppendroff and centrifuge at 8000 rpm for 8 min.
ii. Discard the supernatant then 100 µl of Solution I (ice cold) was poured into tube to get uniform suspension.
iii. Incubate in ice for 30 minutes.
iv. After incubation 200 µl of Solution II was added and inverted microfuse tube several times and mixed all reagents properly.
v. Incubate in ice for 30 minutes.
vi. Then 150µ l of Solution III is added and kept in ice for 5 minutes.
vii. The solution should be mixed in proper way and centrifuse at 10000 Rpm for 10 minutes.
viii. Supernatant is transferred to a fresh microfuse.
ix. After that equal volume of isopropyl alcohol was mixed and incubate at 20 ^{0}C.
x. Then the complete mixture was allowed for centrifugation at 10000 rpm for 10 mins.
xi. Discard the supernatant and further work with pellet.
xii. Next pellet was washed with 70% ethanol.
xiii. Then the pellet was dried at 40^{0}C for completely evaporation of the alcohol.
xiv. After that the pellet was dissolved in TE and load in 1.2% Agarose Gel.

Observation:

Orange colour DNA bands were observed when gel slab was exposed on UV light. Two distinct bands were found. The closer bands indicate released plasmid DNA bands and the farthest was super oil plasmid DNA.

Comment:

The DNA is separated by their molecular weight and bases. The plasmid is observed in gel which indicates that the bacterial cell culture is contained Plasmid.

Restriction Digest of Plasmid DNA

Introduction

Restriction enzyme digestion takes advantage of naturally occurring enzymes that cleave DNA at specific sequences. There are hundreds of different restriction enzymes, allowing scientists to target a wide variety of recognition sequences.

Restriction enzyme digestion is commonly used in molecular cloning techniques, such as PCR or restriction cloning. It is also used to quickly check the identity of a plasmid by diagnostic digest.

Equipment

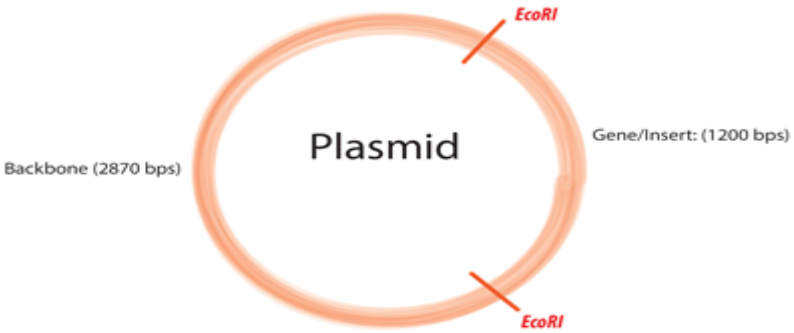
1. Horizontal Gel Electrophoresis chamber

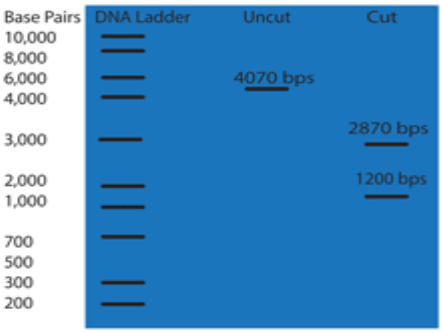
Reagents

1. Plasmid DNA
2. Appropriate restriction enzyme
3. Approrpriate restriction digest buffer
4. Gel loading dye
5. Electrophoresis buffer
6. Micro Pipet and tips

Procedure

1. Select restriction enzymes to digest your plasmid.
2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.
3. In a 1.5mL tube combine the following:
   a. DNA
   b. Restriction Enzyme(s)
   c. Buffer
   d. BSA
   e. DH2O up to total volume

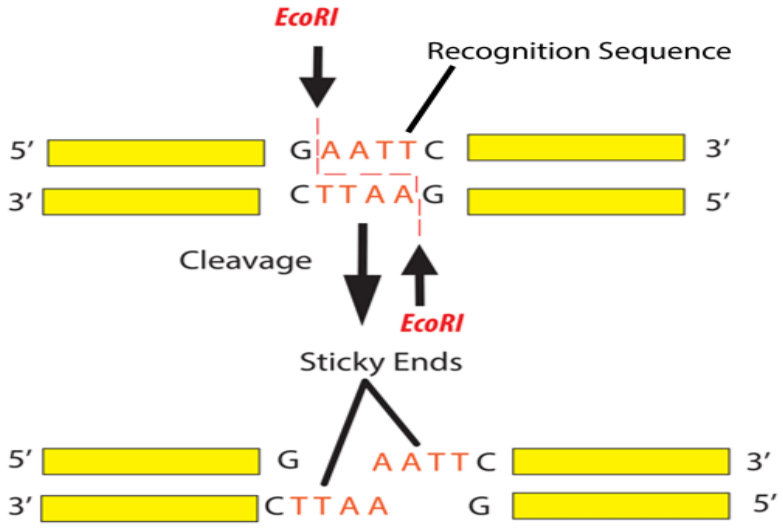




4. Mix gently by pipetting.

5. Incubate tube at appropriate temperature (usually 37 °C) for 1 hour.

6. To visualize the results of your digest, conduct gel electrophoresis.



DNA Extraction from Blood Sample

Introduction

Human DNA can be extracted from all the nucleated cells such as hair, tissue, blood etc. certain sources contain high levels of proteins & many types of secondary metabolites that effects DNA purification, highly purified DNA is essential for molecular studies. Here we followed salting out method to extract large quantities of human DNA from whole blood.

Preparation of Reagents

The reagents were prepared as described below:

a. TKM 1 Buffer / Low salt buffer (500 ml): 0.605 g of TrisHCl (10mM) pH 7.6, 0.372 g of KCl (10 mM), 1.016 g of MgCl2

b. Triton-X (10ml): Added 0.1 ml of 100 % Triton-X to 9.9ml of distilled water. (10 mM), 0.372g of EDTA (2mM) was dissolved in 500ml of distilled water

c. TKM 2 Buffer / High salt buffer (100 ml): 0.121 g of TrisHCl (10mM) pH 7.6, 0.074 g of KCl (10 mM), 1.203 g of MgCl2

d. Cell lysis buffer: 10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5

e. SDS: One gram of sodium dodecyl sulphate was dissolved in 10ml distilled water. (10 mM), 0.074 g EDTA (2mM), 0.467 g of NaCl (0.4 M) was dissolved in 100ml of distilled water

f. 6M NaCl : 8.765 g of NaCl was dissolved in 25 ml of distilled water.

g. TE Buffer: 0.030 g of TrisHCl (10mM) pH 8.0, 0.009 g of EDTA (1mM) was dissolved in 100ml of distilled water

DNA extraction Protocol

1. 900 µl of TKM 1 and 50 µl of 1x Triton-X were added to 300 µl of heparinised blood in an autoclaved 1.5 ml eppendorf.

2. Incubated at 370

3. Cells were centrifuged at 8000 rpm for 3 minutes and the supernatant was discarded. C for 5 minutes to lyse the RBCs.

4. This step was repeated 2-3 times with decreasing amount of 1x Triton-X till

RBC lysis was complete and a white pellet of WBCs was obtained.

5. The pellet was resuspended in 500 µl PBS, followed by addition of 400 µl cell lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5) and 10 µl proteinase K (10 mg/ml stock).

6. The sample was vortexed to dissolve the pellet completely and incubated for 2 h at 56°C in a water bath for lysis. An equal volume of phenol (equilibrated with Tris, pH 8) was subsequently added to the tube and mixed well by inverting for 1 min.

7. The tube was centrifuged at 10,000 g (at 4°C) for 10 min, and the aqueous upper layer was transferred to a fresh tube containing equal volumes (1:1) of phenol and chloroform: isoamyl alcohol (24:1).

8. The tube was mixed by inverting for 1 min and centrifuged for 10 min at 10,000 g (at 4°C). The supernatant was then transferred to a fresh tube, and 10 µl of 10 mg/ml RNase A was added.

9. The sample was incubated at 37°C for 30 min before an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inverting the tube for 1 min and centrifuging at 10,000 g (at 4°C) for 10 min.

10. The supernatant was transferred to a fresh tube, and twice the volume of absolute alcohol was added and inverted gently a few times and chilled at 20°C, followed by centrifugation at 10,000 g at (4°C) for 20 min.

11. The supernatant was discarded, 250 µl 70% ethanol was added, and the pellet was tapped gently, followed by centrifugation at 10,000 rpm for 10 min and decanting the supernatant gently.

12. The pellet was air-dried in a laminar air flow, and the dried pellet was resuspended in 50 µl nuclease-free water or 1X TE buffer and frozen at -20°C or -80°C for storage.

Concentration and Purity Determination

i. A quantitative spectrophotometric assay of DNA was performed using a UV-visible spectrophotometer.
ii. Absorbance was measured at wavelengths of 260 and 280 (A260 and A280, respectively) nm.
iii. The absorbance quotient (OD260/OD280) provides an estimate of DNA purity.
iv. An absorbance quotient value of 1.8 < ratio (R) < 2.0 was considered to be good, purified DNA.
v. A ratio of <1.8 is indicative of protein contamination, where as a ratio of > 2.0 indicates RNA contamination.