CHAPTER 3

MATERIALS AND METHODS

3.1. AMPHIBIAN COMMUNITY STRUCTURE

3.1.1. Field studies: In the present study, the amphibian diversity was analyzed using two different methods. Visual encounter surveys (VES) to prepare checklists and transects. These surveys were done during times of maximum amphibian activity.

a. Visual encounter survey (VES) (Crump, 1971): VES is the most frequently used technique and are the standard methods for sampling pond breeding amphibians (Corn and Bury, 1990; Crump and Scott, 1994). This survey is an appropriate technique for both inventory and monitoring studies (Crump and Scott, 1994). The amphibian diversity in different sites were sampled and checklists of species along with their relative abundance in terms of broad occurrence patterns were prepared. These surveys were conducted during day time and night. Thorough searches were made in all the seasons and all the possible habitats (litter, tree barks, ground cover, bushes, etc.). Boulders were upturned, litters were prodded and the bushes were thoroughly examined. Identification of frogs was done using available literature (Boulenger, 1890, 1920; Daniel, 1963, 1975, 2000; Chanda, 2002) and confirmed by taxonomists.

b. Transect sampling: Randomly located narrow strip transects were laid down, within which portion of the habitats were searched thoroughly for amphibians. Transects were 100m long and 2m wide and portioned into subsections measuring 5m x 2m. Within each subsection every rock was lifted and searched underneath, piece of wood and leaves were turned and the number of individuals of each species was recorded.

3.1.2. Indices

Population census, diversity, evenness, ecological distribution, niche breadth and niche overlap of amphibians at all the study sites were studied using the following formulae (Krebs, 1999).

1. Simpson's index of diversity:

$$1-D = 1-\Sigma(p_i)^2$$

Where (1-D) = Simpson's index of diversity
p_i = Proportion of individuals of species 'i' in the community

2. Shannon-Wiener Diversity Index:

$$H' = \sum_{i=1}^{s} (pi)(\log_2 pi)$$

Where H' = Index of species diversity
S = Number of species
p_i = Proportion of total sample belonging to i^{th} species.

3. Brillouin's diversity:

$$\hat{H} = \frac{1}{N} \log\left(\frac{N!}{n_1! n_2! n_3! \ldots\ldots\ldots}\right)$$

Where $\hat{H}$ = Brillouin's diversity
N = Total number of individuals in entire collection
$n_1!$ = Number of individuals belonging to species 1
$n_2!$ = Number of individuals belonging to species 2
$n_3!$ = Number of individuals belonging to species 3

4. Margalef's equation for evenness:

$$J = \frac{H'}{H_{max}}$$

Where J = Evenness index
H' = Shannon-Wiener diversity index
$H_{max} = \log_2 S$
S = Number of species

5. Levins's Measure of Niche Breadth:

$$B = \frac{1}{\sum p_j^2}$$

Where B= Levins's measure of niche breadth
p_j= Proportion of individuals found in or using resource (microhabitat) state j

6. Levins's measure of standardized niche breadth:

$$B_A = \frac{B - 1}{n - 1}$$

Where B_A= Levins's standardized niche breadth
B = Levins's measure of niche breadth
n = Number of possible resource (microhabitat) states

7. Horn's index of niche overlap:

$$R_o = \frac{\sum (p_{ij} + p_{ik}) \log(p_{ij} - p_{ik}) - \sum p_{ij} \log p_{ij} - \sum p_{ik} \log p_{ik}}{2 \log 2}$$

Where R_o = Horn's index of overlap for species j and k
p_{ij} = Proportion resource i is of the total resources utilized by species j
p_{ik} = Proportion resource i is of the total resources utilized by species k

8. Jaccard index of Similarity coefficient:

$$SC_j = \frac{c}{A - B - c}$$

Where SC_j = Similarity coefficient
c = number of common species
A =total number of species in site A
B = total number of species in site B

9. Coefficient of Community

$$C = \frac{2W}{a - b}(100)$$

Where C = Coefficient of community
a = sum of scores for one site
b = sum of scores for the second site
W = sum of lower scores for each species

3.2. LARVAL STUDY

3.2.1. Sampling

Identification of the amphibians, their eggs and larvae, is an important pre-requisite to an ecological study. During the inventory studies (Chapter 1) it was found that tadpoles of *Bufo stomaticus* belonging to Bufonidae family and *Microhyla ornata* of Microhylidae family were common in the study sites. Therefore, the larval Biology of these two species were scrutinised during monsoon, from June 2004 to September 2004 and June 2005 to September 2005. Eggs and tadpoles of these two species were searched visually and sampled for identification with the aid of D-frame net (40cm x 30 cm). Fifty seven potential aquatic breeding sites, including the permanent and temporary water bodies, were surveyed during the study period at study sites. Data on the natural history of the species, specifically reproductive mode, tadpole habitat and tadpole behaviour were studied.

In the present study, a D-frame net (0.8mm mesh size) was used to know the density of the tadpoles. For each sample, the leading flat edge of the net was placed in the pond bottom perpendicular to the water current and all the specimens were counted in the net. Dip netting is most effective for estimates of abundance in small bodies of water and shallow streams (generally <2m deep) (Heyer *et al.*, 1994).

3.2.2. Morphometric Studies

Tadpoles were caught with the help of dipnet as well as plastic containers were also used to scoop them out. They were then transported to laboratory and examined under stereo microscope (Leica MZ 16 A) for identification. This was further confirmed using available literature (Daniel, 2000; Chanda, 2002). Few of the collected tadpoles were euthenized and preserved in 10% formalin, while some individuals were reared through metamorphosis to ensure a positive identification. Tadpoles were given boiled spinach as food in laboratory. They were reared in plastic tray with about five litres of water from the collecting sites. The tadpoles preserved immediately after the capture and the ones reared in the laboratory, both were used in the description. Nevertheless, no changes were observed in the general shape or oral morphology of the reared tadpoles. The morphological terminology and labial tooth row formula follow Altig *et al.,* (1998). Tadpoles were measured at different stages of development and were identified as described by Gosner (1960). Moreover, the developmental stages of tadpoles were clubbed into 3 groups to study their morphology and morphometry. The first group of tadpoles ranged from stages 25 to 30, the second 31 to 36 and the third 37 to 41 respectively. Finer morphological features were observed under the stereomicroscope (Leica MZ 16A). Morphometric measurements were recorded using calibrated digital caliper (Mitutoyo, Japan) whereas finer measurements were taken with graduated ocular micrometer fitted in stereomicroscope. Drawings were made with a camera lucida.

3.2.3. Scanning Electron Microscopic Analysis

Tadpoles of species *B. stomaticus* and *M. ornata* attains maximum oral disc width by Gosner stage-37, hence oral disc of tadpoles of Gosner's stage-37 were examined under Scanning Electron Microscope. The specimens were prepared as follows: The specimen were cleaned using alcohol and fixed in 4% glutaraldeyde solution for 2 hours at room temperature. Further, they were washed with 0.1M Phosphate buffer and post fixed for 2 hours in a 1% solution of osmium tetroxide. After a wash in 0.1M Phosphate buffer the samples were dehydrated using graded ethanol series. Specimens were dried and mounted

on aluminium stubs with the help of carbon film and sputter coated with gold. Oral anatomy was examined in JEOL-5610LV Scanning Electron Microscope.

3.2.4. Gut Content Analysis

Tadpoles of each species were divided into 2 groups i.e. pre-hindlimb stage and the hindlimb stage. Fifteen tadpoles per stage and per species were examined. For the gut content analysis the tadpoles were collected from the field, killed immediately with MS222 (Tricaine Methane Sulphonate) overdoses and kept in 10% formalin so that the intestinal content does not get digested. Later in the laboratory intestine of the animal was removed, squashed in a small petridish, and a known amount of water was added. Subsequently, Lugol's iodine was also added to presere the plankton samples. A known fraction of the liquefied gut remains was then placed on a Plankton/Neubauer Counting Chamber and analyzed for the food content under a binocular Research Microscope (Leica DMBR).

3.2.5. Controlled Experiments

Controlled experiments were performed to know the pH tolerance of both the species of tadpoles. Tadpoles were kept in waters having different pH ranging from pH 5.5-10.0 and their survival and rate of growth were recorded.

Controlled experiments were also performed in the lab to know whether fishes like *Poecilia sp.* and *Gambusia affinis* predated on eggs and tadpoles of the studied anuran species. In a small aquarium both the species of fishes and eggs as well as tadpoles of both the anurans were kept. The predatory behaviour of the fish was then observed.

3.3. PHYSICOCHEMICAL ANALYSIS

Water from the study sites, were collected and analyzed for various physicochemical parameters as per the treatise, "Standard Methods for the Examination of Water and Wastewater", prepared and published jointly by the American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF). These include temperature, pH, dissolved oxygen (DO), chemical oxygen demand (COD), phosphate-phosphorous ($PO_4^{3-}-P$), nitrate- nitrogen (NO_3^--N), and total solids. Water samples of one litre each were taken from one location per pond. Sampling was done once a week in each month during the study period. Samples were analysed on the same day and these informations were then amalgamated to represent the yearly data. Samples were collected from each site in a clean and contamination free polyethylene containers of two litres volume. They were maintained at 4^0 C during transportation to the laboratory in

order to reduce the growth of microorganisms. For oxygen estimation samples were collected in BOD bottles using dispenser to avoid air contact, and the samples were fixed at the study sites itself. The containers were then labelled indicating the sample number, time and weather conditions alongwith the name of the site.

3.3.1. Temperature

Temperature is basically important for its effect on the chemistry and biological reactions of the organism in water. A rise in temperature of the water leads to the speeding up of the chemical reactions in water and reduces the solubility of gases and also amplifies the tastes and odours. At elevated temperatures the metabolic activity of the organisms increases, requiring more oxygen but at the same time the solubility of oxygen decreased thus accentuating the stress. Temperature is also very important in the determination of various other parameters such as pH, conductivity and saturation levels of gases.

In the present study the ambient as well as the water temperatures were measured at the site using calibrated good grade mercury filled Celsius thermometer.

3.3.2. pH

pH can be used as an indicator of pollution in an aquatic system. Natural waters usually have a pH between 6.0 and 8.5. However, pH of water gets drastically altered in time, because of exposure to air, biological activities and temperature changes. The pH of a water body can change widely by the addition of industrial effluents and municipal sewage from the settlements including the non point sources. Waters, with pH value of more than 9.6 or less than 4.5 becomes unsuitable for most life forms and also for most other uses.

In the present study pH was measured electrometrically using a hand held pH meter.

3.3.3. Dissolved oxygen (DO)

Dissolved oxygen is one of the most important parameters in water assessment. It reflects the physical and biological processes prevailing in the waters. Its presence is essential to maintain the higher forms of biological life in the water. Low oxygen in the water can kill fish and many other organisms in the water because they have specific requirements of oxygen. Low oxygen concentrations are generally associated with heavy contamination by organic matter. Oxygen saturated waters have a pleasant taste while water with less dissolved oxygen have insipid taste. The analysis for DO is a key test in water pollution and waste treatment process control (APHA, AWWA 1998). In the present study Winkler's Modified

Method as described in APHA, AWWA (1998) was employed for determining Dissolved oxygen.

Principle: Oxygen oxidizes Mn^{2+} to a higher state of valence under alkaline conditions and that manganese in higher states of valence is capable of oxidizing I to I^2 under acidic conditions. Thus the amount of I^2 released is equivalent to the dissolved oxygen originally present. The iodine is measured with standard sodium thiosulphate solution and interpreted in terms of dissolved oxygen.

Procedure: Samples were collected carefully avoiding agitation and contact with air in narrow mouth glass-stopper BOD bottles of 300mL capacity. They were allowed to overflow 2-3 times its volume. These samples were then fixed in order to prevent biological activity, which can radically change the oxygen values. Fixation was done with 1mL of each manganous sulphate ($MnSO_4$) and alkali-iodide-azide solution. The bottles were stoppered and inverted a few times for proper mixing, and were then transported to the laboratory for analysis. When the precipitate of manganous hydroxide had settled to half the volume, one mL of concentrated sulphuric acid was added. When the dissolution was completed 200mL of the sample was titrated against 0.025M sodium thiosulphate using starch as the indicator. The end point was determined by disappearance of the blue colour.

3.3.4. Total Solids

Total solids include both the dissolved and suspended solids. The density of total solids determines the flow of water in and out of the cells of organisms. These solids are essential at certain concentration to maintain aquatic life. However high concentration of total solids increase water turbidity and this in turn decrease the light penetration.

Procedure: 100 ml of sample is taken in an oven dried preweighed silica crucible. The sample is evaporated to dryness at 103^0C to 104^0C for 24 hrs. The crucible is then cooled at room temperature and the final weight is recorded.

3.3.5. Chemical oxygen demand

Wastewater quality indicator such as the chemical oxygen demand (COD) is essentially laboratory tests to determine whether or not a specific wastewater will have a significant adverse effect upon aquatic flora and fauna. COD test measures the oxygen demand of biodegradable pollutants plus the oxygen demand of non-biodegradable oxidizable pollutants. In environmental chemistry, the chemical oxygen demand (COD) test is commonly used to indirectly measure the amount of organic compounds in water. Most

applications of COD determine the amount of organic pollutants found in surface water (e.g. lakes and rivers), making COD a useful measure of water quality. It is expressed in milligrams per liter (mg/L), which indicates the mass of oxygen consumed per liter of solution.

Principle: Most types of organic matter are oxidized by a boiling mixture of chromic and sulfuric acids. A sample is refluxed in strongly acidic solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ is titrated with ferrous ammonium sulfate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent. Keep ratios of reagent weights, volumes, and strengths constant when sample volumes other than 50 mL are used. The standard 2 hr reflux time may be reduced if it has been shown that a shorter period yields the same results. Some samples with very low COD or with highly heterogeneous solids content may need to be analyzed in replicate to yield the most reliable data.

Procedure: To 50mL sample, 1g of Mercury sulfate, several glass beads, and 5.0 mL sulfuric acid reagent is added so as to dissolve $HgSO_4$. Cooling is done while mixing, to avoid possible loss of volatile materials. To this, 25.00 mL 0.04167*M* $K_2Cr_2O_7$ solution is added and mixed. Further, 70 mL of sulfuric acid is slowly mixed by continuous swirling. The open end of condenser is covered with a small beaker to prevent foreign material from entering refluxing mixture and refluxing is done for 2 hr. After the reflux, the condenser is disconnected and the mixture is diluted to about twice its volume with distilled water. After cooling at room temperature, titration is done with 0.1N ferrous ammonium sulphate, using 0.10 mL (2 to 3 drops) of ferroin indicator. The end point of the titration is the first sharp colour change from blue-green to reddish brown that persists for 1 min or longer. In the same manner, blank containing the reagents and a volume of distilled water equal to that of sample is refluxed and titrated.

3.3.6. Phosphate- Phosphorous ($PO_4^{3-} - P$)

Phosphorous occurs in natural waters and in wastewater almost solely as phosphates. They occur in solution, as particles or detritus or in the bodies of aquatic organisms (APHA, AWWA 1998). Various forms of phosphates arise from a variety of sources. Large quantities of phosphates are added when the water is used for laundering or other cleaning because these materials are major constituents of many commercial cleaning preparations. Many heavy-duty synthetic detergent formulations contain 12-13% phosphorous or over 50%

polyphosphates. The use of these materials as substitute for soap has greatly increased the phosphorous content of domestic waste water (Sawyer *et al.*, 1994)

Orthophosphates applied to agricultural land as fertilizers are carried into surface water with storm runoff. Organic phosphates are formed primarily by biological processes. Most of the inorganic phosphorous is contributed by human wastes as a result of the metabolic breakdown of proteins and elimination of the liberated phosphates in the urine (Sawyers *et al.*, 1994). In the present study the total phosphate-phosphorous of the water was estimated using the Stannous Chloride method.

Principle: Molybdophosphoric acid is formed and reduced by stannous chloride to intensely coloured molybdenum blue. The absorbance of light by this blue colour is measured at 690nm to calculate the concentration of phosphates

Procedure: To 50 ml of sample, 2 ml of ammonium molybdate reagent and 5 drops of stannous chloride is added and thoroughly mixed. After10 minutes but before 12 minutes the colour is measured photometrically at 690nm.

3.3.7. Nitrate-Nitrogen ($NO_3^- - N$)

Very few mineral sources of nitrate exist in nature. The most important source of nitrate is the biological oxidation of organic nitrogenous substances, which come in sewage and industrial wastes or produced indigenously in the waters. Domestic sewage contains very high amounts of nitrogenous compounds. Run-off from agricultural fields is also high in nitrate. Atmospheric nitrogen fixed into nitrates by the nitrogen fixing organisms is also a significant contributor to nitrates in the water. High amount of nitrates is useful in irrigation but its entry into the water resources increases the growth of nuisance algae and triggers eutrophication.

Principle: In the present study nitrate was estimated using the Cadmiun Reduction Method. This method is based on the principle that nitrate is reduced almost quantitatively to nitrite in the presence of cadmium (Cd). It uses commercially available Cd granules treated with copper sulphate ($CuSO_4$) and then packed in a glass column. The nitrite produced thus is determined by diazotizing with sulphanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye that is measured colorimetrically. Initially the cadmium granules have to be activated by first washing them with 6N HCl and then rinsing with water. These are then swirled with 2% $CuSO_4$ solution

until the blue colour partially fades. This is decanted and the process is repeated till a brown colloidal precipitate begins to from. Finally it is flushed gently with water to remove the copper. The reduction column is then filled with the Cu-Cd granules. This is then washed with diluted NH_4Cl-EDTA solution. And finally activated by passing through it 100ml of a solution composed of 25% 1.0mg NO_3^- – N/L standard and 75% NH4Cl-EDTA solution.

Procedure: To 25 ml sample, 75 ml of NH_4Cl-EDTA solution is added and mixed. This mixed sample is then passed through the reduction column and collected at a rate of 7 to 10ml/min. First 25 ml are discarded and the rest is collected. And then to 50 ml of this sample is added 2 ml of the colour reagent and mixed. Between 10min and 2 hrs after reduction the absorbance is measured at 543 nm against distilled water blank. Sample concentrations can then be computed by directly comparing with the standard curve, which is obtained by plotting absorbance of standards against NO_3^- – N concentrations.

3.4 METHOD FOR HEAVY METAL ANALYSIS

3.4.1. Principle

Atomic absorption methods involve conversion of the sample into an atomic vapour and measurement of the absorbance of this vapour at a specific wavelength, is the characteristic of the analyte element (Sir Walsh, 1982).

3.4.2. Test Organisms

A total of 15 Adult *Euphlyctis cyanophlyctis* of both sexes were collected from each study sites, so as to comprehend the bioaccumulation of selected heavy metal in these animals. The frogs were then sexed and anaesthetized with ether, individually weighed, and snout to vent length measured. After anesthetization, the belly was excised and the tissues to be analyzed were removed, digested and heavy metals were assessed using atomic absorption spectrophotometer. The metals analyzed in the current study were Nickel (Ni), Cadmium (Cd) and Chromium (Cr) (Refer materials and methods for detail).

3.4.3. Method of tissue digestion

Heavy metal such as Ni, Cd and Cr were chosen to study due to their widespread contamination and long persistence. To analyze heavy metals, each tissue sample *viz.* liver and kidney were dried at 45°C for 48 hours. Nitric acid digestion methods were applied for the digestion of tissue. This approach was partly modified from that of Hseu (2004). 0.5 gm sample was digested with 5 ml of concentrated HNO_3. The tissues were left in the nitric acid for 24 hr and then were put in a hot plate, at 80°C for 2-3 hrs. After that, the temperature

was raised to 140-150°C and the tissues remained for 4-5 hrs, until a clear solution was obtained. Further, 5 ml of concentrated HNO_3 was added to the sample and digestion occurred until the volume was reduced to 1 ml. The interior walls of the tube were washed down with a deionized water and the tube was swirled throughout the digestion to keep the wall clean and prevent the loss of the sample. After cooling, the final volume was made to 10 ml by adding deionized water. The chemical used for sample dissolution was of analytical grade.

Concentrations of these heavy metals were assessed using Perkin Elmer atomic absorption spectrophotometer with air - acetylene flame employing the nitric acid method. The metal concentrations were calculated in microgram per gram, dry tissue weight. Standard ranges used for spectrophotometry are as follows: Nickel 2 – 10 µg/L, Cadmium 0.2 – 2 µg/L, Cr 0.2 – 2µg/L and the stock solution was serially diluted in metal free deionized water.

3.4.4. Heavy metal analysis for Water samples

In the similar way water was collected from the study sites and was analyzed for heavy metals. After collecting the water samples it was acidified (pH> 2) using Conc. HCl. These samples were then directly analyzed using atomic absorption spectrophotometer. Metal concentrations were calculated in microgram per ml of water sample (µg / mL)

3.4.5. Expression of Results

The concentration of metals in tissue is reported in µgram of metal per gram, dry weight of tissue. It is calculated as follows:

Cm = (C x V x dil'n) / Wd

Where Cm = concentration of element in the tissue in µg/g dry weight

C = concentration of element in the digest in µg/mL

V = volume of the digest in mL

Wd = weight of dry sample in g

Dil'n= sample dilution (if any)

3.5. METHOD FOR DIAGNOSING CHYTRIDIOMYCOSIS

3.5.1. Collection of specimen

Anurans belonging to different families were collected during monitoring programme of amphibian population in Vadodara district, Gujarat. The study was conducted during 2005. Species from four different families' *viz.* Dicroglossidae, Bufonidae, Microhylidae and

Rhacophoridae were collected from both polluted and unpolluted sites and were then diagnosed for the disease using histology methods.

3.5.2. Principle of histology: Tissues are too thick to allow light to be transmitted through them. Thus, these tissues are sliced into very thin sections provided they are first processed to prevent cell damage. The processing involves a series of steps; fixation, dehydration, embedment and subsequent sectioning with a microtome. Fixing cells with formaldehyde will preserve the general organelle structure of the cell. However, formaldehyde, has the potential to further react with the staining procedure which is used later in the process. Consequently, any remaining fixative is washed out by placing the tissue in running water for 1-2 hrs. Before embedding the tissue in paraffin all traces of water must be removed, as water and paraffin are immiscible. The removal of water is known as dehydration. The dehydration process is accomplished by passing the tissue through a series of increasing alcohol concentrations. After dehydration, the tissues can be embedded in paraffin and sectioned with the help of microtome. This is then stained with the help of hematoxylin and eosin.

3.5.3. Procedure for histology: Strips of skin from the pelvic region were undertaken for the study of chytridiomycosis, as the chytrid fungus invades only the stratum corneum and stratum granulosum (Berger *et al.*, 1999). Tissues (skin) were preserved in 10% formalin. These tissues were then washed and were further dehydrated. The dehydration process was accomplished by passing the tissue through a series of increasing alcohol concentrations. The tissues were then transferred sequentially to 30%, 50%, 70%, 80%, 90%, 95%, and absolute alcohols for about fifteen minutes each. They were then placed in a second 100% ethanol solution to ensure that all water is removed. After dehydration, clearing of the tissues was done by moving the tissue into a 50:50 mixture of absolute ethanol:toluene for two hours. This is then followed by placing the tissues in pure toluene and then into a mixture of toluene and paraffin (50:50). The blocks were then transferred to pure paraffin in the oven at 58-60 for 20 minutes. During this time the tissue block is completely infiltrated with melted paraffin. Subsequent to infiltration, the tissue is placed into an embedding mold and melted paraffin is poured into the mold to form a block. The blocks are allowed to cool and are then used for sectioning. The solidified blocks were trimmed to 6 to 8 micron thickness and taken on the slide coated with egg albumin.

These slides with the skin sections were first placed in xylene for 15 minutes and were then rehydrated by placing the tissue sequentially to 100%, 90%, 70%, 50%and 30% alcohol for about fifteen minutes each. The section were then stained with haematoxylin for 5 minutes

and then washed in running tap water for 30 minutes so as to remove the excess stains. This is followed by staining the sections with eosin for 5 minutes and subsequently dehydrating, by transferring to 30%, 70%, and 90% alcohol for 5 seconds each. After drying, the slides were cleared in Xylene twice for 10 minutes. These cleared tissue sections were permanently mounted with DPX. The permanently mounted sections of the tissues were observed under Leica DMBR Advanced research microscope fitted with calibrated graticule, for further histological evaluations.

3.5.4. Methods Used to Confirm the Disease (by Diana Mendez)

The sequences of steps used to make a diagnosis of chytridiomycosis are:

1. Looking for focal hyperplasia of the superficial epidermis (to do a particularly thorough search for Bd).
2. Identifying possible zoosporangia - clear oval or roughly round spaces - in the stratum corneum and stratum granulosum (top layers of epidermis) only.
3. Looking specifically for distinct walls around these spaces. At this stage ignoring the contents.
4. Looking for structures of the suspect sporangia that confirm the identification as Bd = (a) discharge tube (b) internal septum.
5. Checking the size - usually less than 15 microns diameter.
6. Look inside the zoosporangia for internal structures of the developing zoospores:
    a) early stage = single dense blue ball
    b) division occurring = several dense blue structures, separated by fine pale lines.
    c) zoospores formed = multiple small blue bodies (max 2 micron diameter), crowded in zoosporangium
    d) empty zoosporangia = occasionally a single zoospore that has not escaped may be seen. Empty zoosporangia may also change shape and look like flattened ovals.
    e) empty zoosporangia that have expelled zoospores but have been colonized by bacteria = with a fine blue cloud or collection of dust-like particles which are colonies of bacteria that stain with haemotoxylin and appear like a bluish cloud, but on oil immersion, individual bacteria (usually rods) can be seen.

3.5.5. Confirmation of the Results: The results were further confirmed by sending the images to expert like Rick Speare and Diana Mendez (James Cook University, Queensland).