CHAPTER II

LITERATURE REVIEW

Xenoestrogens

Xenoestrogens are synthetic substances that differ from those produced by living organisms and imitate or enhance the effect of estrogen. These substances are part of a heterogeneous group of chemicals that are hormone. Xenoestrogens from a variety of sources may have a cumulative effect upon living organisms and act via different modes of action, but the estrogen receptor–mediated mechanism is the most important one (Kime et al., 1995). Estrogenic compounds are characterised by their ability to bind to and activate the estrogen receptor (ER) by entering the cell. They differ from phytoestrogen, mycoestrogens, and pharmacological estrogens (Mellanen et al., 1996).

Sources of xenoestrogens

Xenoestrogens are released into environment from industry, agriculture, and household, accompanied by tons of unintended byproducts. Regardless of the source of origin, substantial amounts of these chemicals end up in the aquatic environment due to physico-chemical, hydrologic and atmospheric process (Arukwe et al,1998, Ahlborg & al, 1995). Among xenoestrogens are synthetic steroids such as those used in the contraceptive pill, some organochlorine pesticides (dichlorodiphenyltrichloroethane; DDTs, hexachlorocyclohexanes; HCHs), surfactants and detergents (alkylphenol polyethoxylates; APEs), plasticizers (phthalates), polychlorinated biphenyls; PCB, and some natural chemicals such as phytoestrogens and mycoestrogens (Arukwe et al., 1998).

Organochlorine pesticides, including DDT (dichlorodiphenyltrichloroethane) and DDE (dichlorodiphenyldichloroethylene), represent an important class of xenoestrogens which have the general property of being lipophilic and persistent in environment. DDE is a breakdown product of DDT that can cause a wide variety of health problems in humans and wildlife. In humans, it may cause cancer and damages to the liver and to the nervous and reproductive systems. In birds it can also result in eggshell thinning. DDT-related compounds bioaccumulate in the tissues of fish, birds and mammals and biomagnify in the ecosystem. DDE is the most persistent of all the DDT metabolites (Miller et al., 1998).

Alkylphenol comprises a large group of chemicals which has been in use for over 40 years. They are classed as being non-ionic surfactants, which have many and varied industrial uses, most notably as industrial detergents, and in the wool industry. They are also used in some household detergents (Arukwe et al., 1998). Production of alkyphenolic compounds is millions of kilograms annually, of which some 60% find their way into the aquatic environment, which are recognized as being harmful to life (Miller et al., 1998).

Phthalates are plasticizers which are a family of industrial chemicals that are used as plastic softeners or solvents in many different products such as deodorants, car seats, hair spray, IV bags, perfume, vinyl flooring, wallpaper, and siding all contain phthalates (Mellanen et al., 1996).

Toxicity of xenoestrogens to marine organisms

Most evidences for xenoestrogenic effect in wildlife have come from animals that live in or are closely associated with the aquatic environment. Freshwater and marine environments act as repositories for large volume discharges of thousands of

different chemicals. For wildlife associated with these environments, major routes of chemical exposure include the skin and gill surfaces, as well as through the diet. In addition, many aquatic (and some semi-aquatic) animals deposit their eggs or embryos into the water thus potentially expose their vulnerable life stages directly to cocktails of xenoestrogens.

It has recently become more apparent that several effects have been observed from fish exposure to toxicants. These include the inhibitions of oocyte development and maturation, increase of follicular atresia from both yolk and previtellogenic oocytes, abnormality of yolk deposition and formation within oocytes, and abnormality of egg maturation and production (Arukwe et al.,1998, Kime et al., 1999). Several studies also reported the effect of xenoestrogen-induced reproduction disturbances in fish which could change male secondary sex morphological characters (Davis et al., 1992), caused higher eggs and larvae mortality, reduced number of eggs produced (Waring et al., 1996), decreased hatching rate, and malformed embryos (Dethlefsen et al, 1996). A large research effort has focused on the development of effective methods for monitoring xenoestrogen contamination.

Monitoring of xenoestrogen problem

Methods for monitoring environmental problem can be divided into 2 distinct categories; (1) the detection of pollutants and their quantification in physical and biological mediums, (2) the evaluation of the effects of pollution on living organisms, either at the individual level or at the level of populations and/or communities (Langdon et al., 1997).

The detection and quantification of pollutants method based on chemical analysis which is currently highly improved and able to measure the extent and level of some environmental contamination by using a limited number of sample taken from various matrices (water, soil, sediments, plants, animals etc.). However, the use of such method is not always possible because of the properties of the substances being studied making no method is able to quantify all the contaminants present in a sample. Moreover, the analytical techniques do not allow to evaluate the effects of the presence of contaminants on living organisms or the health of ecosystem.

For the biological approach, methods based on quantitative and qualitative observation of living organism are used. The study of certain species or groups of species, the presence (or absence) and/ or abundance provide information about environmental quantity. Another one is measurement, in individuals from natural environment, of molecular, biochemical, cellular, or physiological parameters. Such indicators are called biomarkers (Arukwe et al., 1998).

Biomarker is becoming an important tool for monitoring programs. There are many definitions of biomarker. Typically, biomarkers are defined as quantitative measures of changes in the biological system that respond to either (or both) exposure to, and /or doses of, xenobiotic substances that lead to biological effects. The use of biomarker presents the advantage of an integrated evaluation in time and space of bioavailable pollutants, not only term of presence, but also in relation to the effects of these products on animal, plant, and microbial populations. Among several biomarkers for exposure assessment in free-living organism and in environmental pollution, vitellogenin and zona radiata protein are potential biomarkers widely used for xenoestrogen contamination.

Vitellogenin and Zona radiata protein

Vitellogenin (VTG), a phospholipoglycoprotein, is a precursor of egg yolk proteins. In oviparous animals, VTG is normally synthesized in the liver upon induction by estrogen. In general, VTG undergoes posttranslational modification including lipidation, glycosylation, and phosphorylation before being secreted into blood. The occurrence of VTG is specific to the female, also known as the female-specific plasma protein. VTG is a complex calcium-bindng phospholipoglycoprotein. The VTG of a number of fish species consists of 2 to 3 subunits. Such as 3 subunits in goldfish (*Carassius auratus*) (de Vlaming et al., 1980), 2 in grouper (*Epinephelus malabaricus*), (Utarabhand et al., 1996). Several studies on VTG in teleost fish are summarized in Table 2.1.

When VTG is transported through the blood to the ovary and incorporated into the oocytes by specific receptors, it is cleaved in to the yolk protein lipovitellin and phosvitin. These yolk materials accumulated in oocyte during embryogenesis is an important process for the success of reproduction. Yolk is stored in oocyte until the late stages of oogenesis before mobilized into the embryo to provide the nutrients for embryogenesis. In general, lipovitellins are glycolipophosphoprotein with a very low content of phosphorus while phosvitins are rich in phosphorus but lack lipid and carbohydrate.

A number of investigators have attempted to characterize lipovitellin and phosvitin from the ovaries of a variety of teleost species. Such as Campbell and Idler (1980) demonstrated that trout lipovitellin has a molecular weight of 300 kDa and phosvitin have a molecular weight about 43 and 19 kDa. De Vlaming (1980) demonstrated that the 2 subunits of lipovitellin from goldfish has a molecular weight 105-110 kDa and 19-25 kDa by using SDS-PAGE.

Zona radiata proein (ZRP), a glycoprotein, is a precursor of egg envelope proteins surrounding the animal egg. ZRP of fish eggs also plays a significant role in the reproductive and developmental processes; firstly as an interface between the egg and sperm and secondly as an interface between the embryo and its environment. The egg envelop is a major structural determinant of the eggshell in fish. It is often referred to as zona radiate protein because of its striated appearance under the light microscope (Augustine et al., 2003) (Fig 2.1). This layer is a much thinner, transparent, extracellular, envelope that lies immediately outside the plasma membrane in the eggs of placental mammals (Sugiyama et al., 1996).

The fish egg envelope has been conventionally regarded as a so-called primary egg envelope that is synthesized in oocytes (Chang et al., 1997). However, the origin and the number of the egg envelope proteins in fish have appeared to vary amongst species and recent studies have report that precursor proteins of the egg envelope subunits of many fish species are synthesized in the liver under influence of estrogen (Lee et al, 2002, Augustine et al, 2003). ZRP is comprised of 3 – 4 protein monomers distinctly conserved glycoprotein with molecular weight in the range of 50 – 60 kDa (Oppen – Berntsen et al., 1992.). ZRP in Atlantic salmon (*Salmon salar*), Cod (*Cadus morhua*), and rainbow trout *(Oncorhynchuc mykiss*) exist as 3 monomers known as α, β, and γ.

Arukwe et al. (197b.) studied of ZRP from plasma of juvenile Atlantic salmon by treating with 4-nonylphenol using SDS-PAGE, western blot analysed. The molecular mass of ZRP α, β, and γ were found to be 60, 55, 50 kDa, respectively. Oppen – Berntsen et al. (1992) studied of ZRP from eggshell extracts of cod (*Cadus morhua*) using SDS-PAGE analysed. The molecular mass of ZRP were found at 78, 54, 47 kDa, respectively. Sugiyama et al. (1996) analyzed ZRP from solubilization of

egg envelop of medaka (*Oryzias latipes*) using SDS-PAGE analysed. The solubiliaed materials consisted of two major groups of the inner layer supunits, those having the molecular weight of 74-76 and 49 kDa.

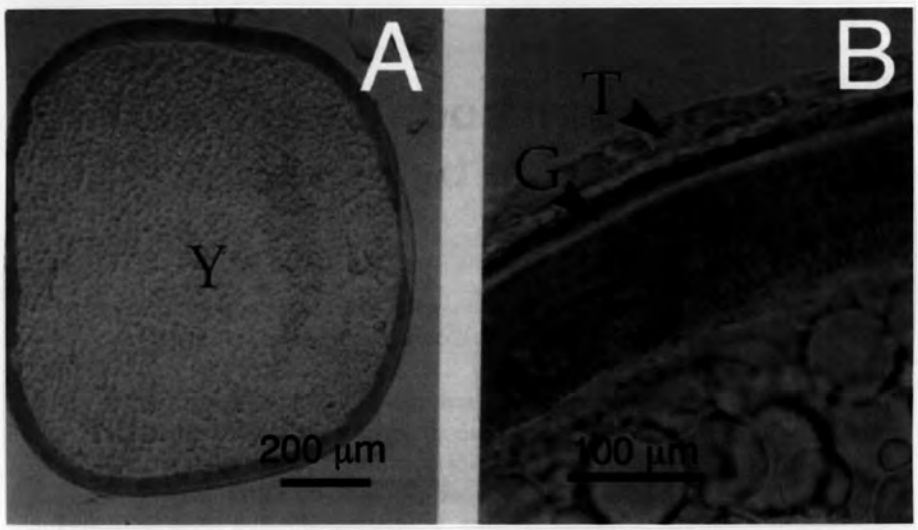

Figure 2.1 Immunohistochemical staining of cod (Gadus morhua) ovarian follicle cell and oocyte (Augustine et al., 2003).
(A) Section of whole oocyte (yolk material ,Y)
(B) The follicle cells (theca, T, zona radiata protein, Zr and granulosa, G)

The structure of the growing ovarian follicle is remarkably similar in most fish. The developing oocyte is located in the centre of the follicle and is surrounded by steroid producing follicle cells. The follicle cell layer generally consists of an inner sublayer, the granulosa cell layer, and one or two outer sublayers of theca cells. The

theca and granulosa cell layers are separated by a basement membrane. Between the surface of the oocyte and the granulosa cell layer, there is a cellular layer which is the *zona radiata* or eggshell. During oocyte development, ZRP are sequestered from circulating plasma and deposited in the oocyte. At the same time, the oocyte is being filled with yolk proteins (lipovitellin, phosvitin), derived from VTG. Both of these proteins (VTG and ZRP) are the important constituents of the mature oocyte. They are synthesized in the fish liver under endocrine regulation through the hypothalamic-pituitary-gonadal-liver axis.

Vitellogenesis and Zonagenesis

The development of the eggshell (oogenesis) in teleost fish can be divided into two main parts: oogenetic growth and final maturation of the ovum. The oogenetic growth phase consists of two different processes, zonagenesis and vitellogenesis. Vitellogenesis and zonagenesis are the hepatic synthesis of egg yolk protein precursor (VTG) and eggshell (ZRP) that regulated by estradiol-17β (E_2) or estrogen. Pituitary gonadotropins (GtHs) and ovarian steroid hormones regulate oocyte growth and maturation in teleosts and other vertebrates. As explained by simplified general model, environmental changes such as water temperature and photoperiod provide the cues to the central nervous system that triggers the maturation processes. In response, the hypothalamus secretes gonadotropin-releasing hormone (GnRH). As the central regulator of hormonal cascades, GnRH stimulates the release of GtHs (GtH I & II) from the pituitary, GtH I is involved in vitellogenesis and zonagenesis, while GtH II plays a role in final oocyte maturation and ovulation. GtH I also stimulates the follicle cells to synthesize estrogens or estradiol-17β (E_2). Estradiol enters the liver cells by diffusion and is retained in target cells by high affinity binding to a specific receptor

protein called estrogen receptor (ER) and stimulates the production of VTG and ZRP (Fig 2.2).

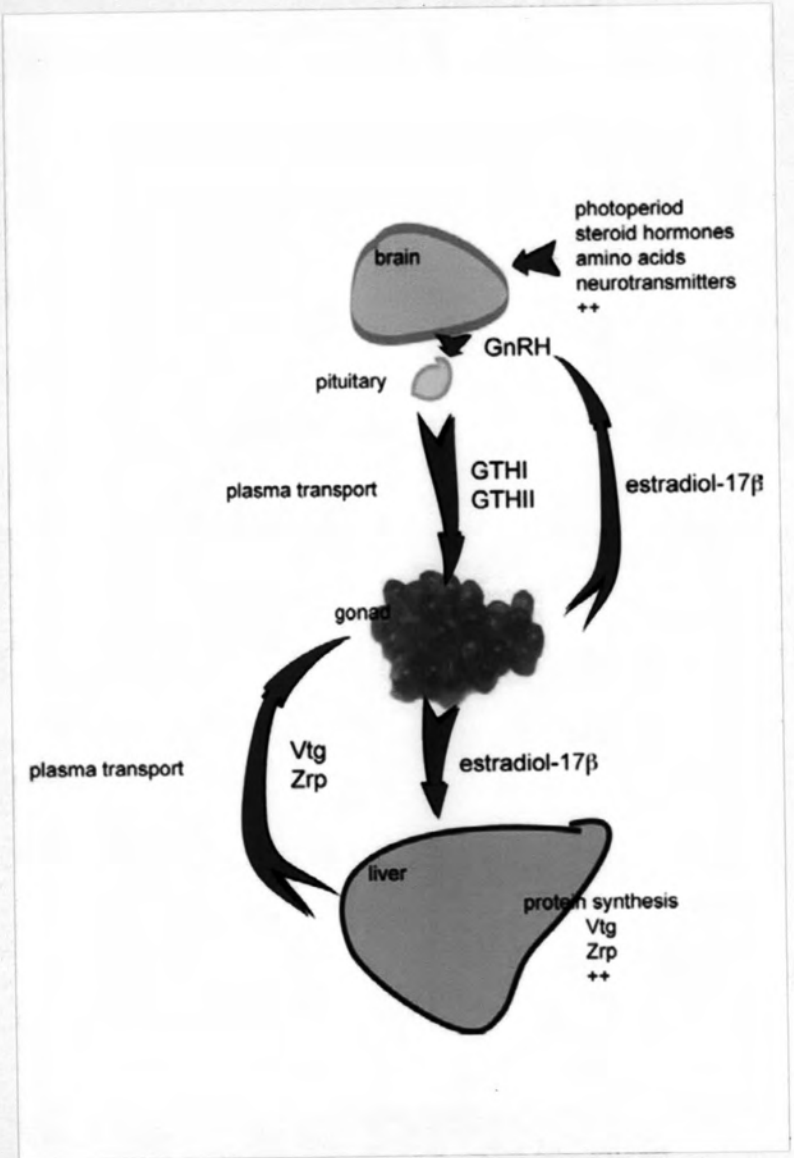

Figure 2.2 The hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in female teleosts (Arukwe et al,1998).

Determination of VTG and ZRP

VTG and ZRP are relatively easy to measure in blood plasma by using immunosorbent assay techniques such as enzyme–linked immunosorbent assay (ELISA). This immunoassay has been developed to monitor the concentration of VTG in plasma and the yolk in ovary. Immunodiagnosis was particularly useful because high sensitivity and specificity. Immunological method can be simplified to obtain results quickly at relatively low cost (Sithigorngul et al., 2002). In developing a bioassay for xenoestrogen effects, antibodies against fish VTG and ZRP have been produced for the development of ELISA assay. Several polyclonal antibodies against fish VTG and ZRP have been developed for this purpose such as polyclonal antibodies against VTG in carp *(Cyprinus carpio)* and perch *(Perca fluviatilis)* (Hennies et al,2003), fathead minow (*Pimephales promelas*) (Park et al, 1999) brown trout (*Salmo trutta*) (Sherry et al,1999) but only a few monoclonal antibodies have been produced. For ZRP, polyclonal antibodies against ZRP from a small number of fish have been developed. Additionally, monoclonal antibodies against ZRP have only recently become available (Berg et al., 2002).

Monoclonal antibodies

Antibodies (also known as immunoglobulin) are a class of protein molecules produced by B lymphocytes of the adaptive immune system which act as flexible adapters between the infectious agents and phagocytes (Roitt, Brostoff and Male, 1985). Antibodies are produced and appear in the serum when an animal encounters an antigen either by infection or by deliberate injection. The chemical and physio-chemical properties of these highly heterogeneous protein molecules are called polyclonal antibodies. However, their heterogeneities of size, structure, charge and

biological activity have made isolation and purification a challenging problem for immunological analysis. Antibodies used in immunological test systems are powerful tools for the specific detection and identification of several proteins. Monoclonal antibodies (MAbs) are important reagents used in biomedical research, these antibodies are produced by cell line or clones obtained from animal that have been immunized with the substance that is the subject of study. The cell line is produced by fusing B cells from immunized animal with myeloma cell (Köhler and Milstein, 1975). To produce the desired MAb, the cells must be grown in either of the two ways: the injection into the peritoneal cavity of a suitably prepared mouse (the *in vivo*, or mouse ascites method) or *in vitro* tissue cultures.

The general principle of the antibody producing hybridoma by tissue culture is shown in Figure 3. They consist of the fusion of a non-secreting myeloma tumor cell and an antibody-producing B-cell. The fused cell retains the immortality of the myeloma cell line and also continues to secrete the antibody produced by the B cell. The fused cell (hybridoma) may then be maintained in tissue culture. This method could be utilized to produce monoclonal antibodies with specificity for any antigen (Steward et al., 1984).

Monoclonal antibody production

The most monoclonal antibodies used in research, medicine and biotechnology are of mouse origin (Vetterlein et al., 1989).

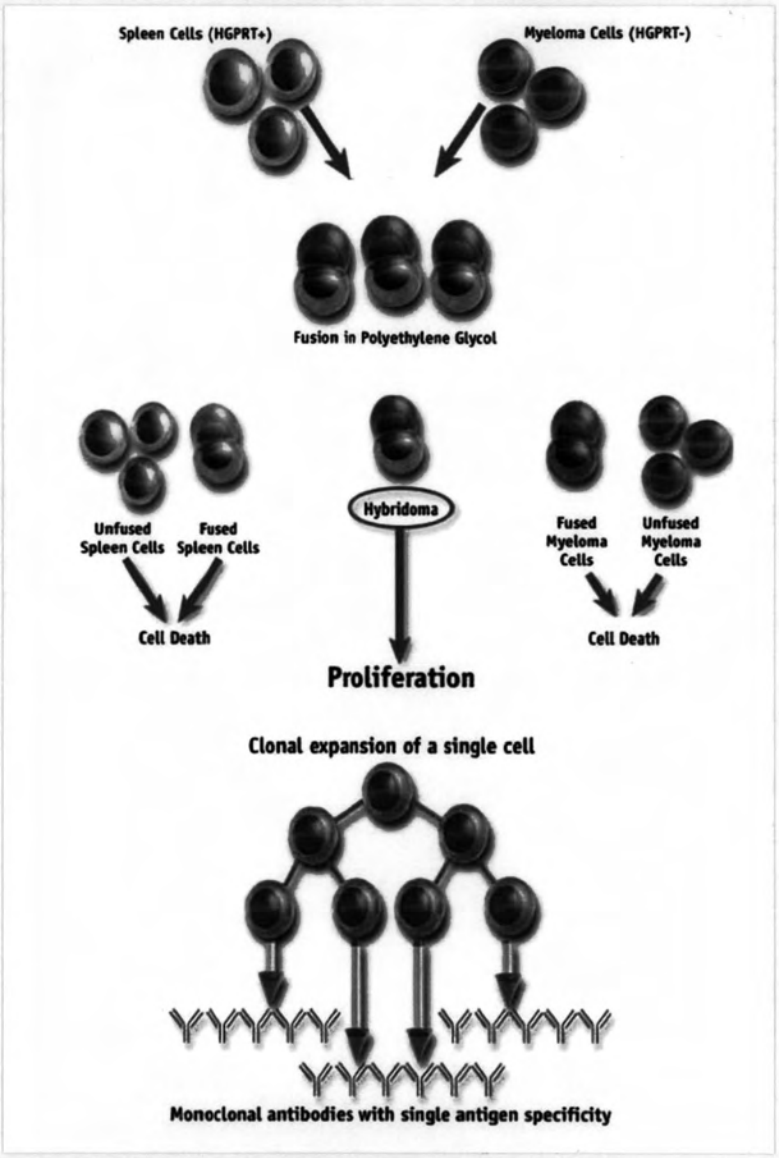

Figure 2.3 The principle of the production of an antibody-

Producing hybridoma (http://images.encarta.msn.com/targetsA.gif)

For immunization, primary injection of antigen emulsified in complete Freund's adjuvant is injected intraperitoneally (other injection site such as intramuscular hind legs and hind foot-pads are also commonly employed). Secondary injections are given using incomplete Freund's adjuvant at 2-3 week intervals until an antibody response is detected by assay for serum antibodies. To ensure an optimum antigenic response, a final intravenous injection of the antigen in microgram amounts is often given 2-3 days before spleen or lymphoid cells are removed.

Myeloma cells lacked the purine salvage enzyme hypoxanthine guanosine phophoribosyl transferase (HGPRT) are selected for the fusion. This selection is achieved by exploiting the fact that $HGPRT^-$ cells are able to grow in Hypoxanthine/ aminopterin/ thymidine (HAT) selective medium. The hybridization of these myeloma cells and the spleen with high concentrations of polyethylene glycol (PEG). The fused cells are cultured in a selective media (HAT medium). In this medium, the tumor cells are killed, the normal, non-fused cells die after a short period in culture and the hybridomas survive. The reason for the selective effect of the HAT medium will be briefly described and seen in Figure 4. Cells have two ways of producing nucleic acid: (a) *de novo* synthesis pathway and (b) the salvage pathway in which nucleotides from degraded nucleic acids are utilized by a process which requires the enzyme HGPRT. In the presence of aminopterin, *de novo* synthesis is blocked but normal cell can survive by using the salvage pathway and the nucleotides in the HAT medium. However, the $HGPRT^-$ tumor cells die in the presence of aminopterin because they can use neither *de novo* synthesis nor the salvage pathway. The hybridoma cells survive because they have HGPRT derived from genes received from the normal spleen cell parents (Steward et al., 1984).

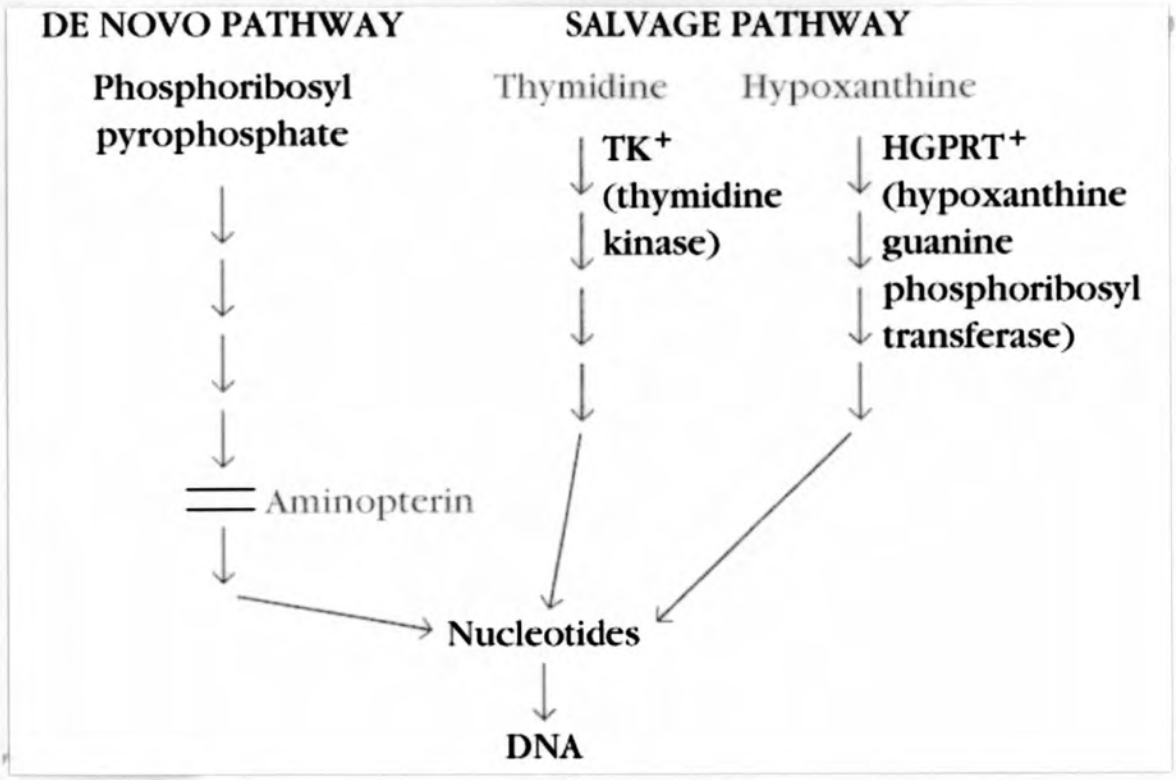

Figure 2.4 Nucleotide synthesis : *De novo* and salvage pathway.

(http://immuneweb.xxmc.edu.cn/images/figure04-21.jpg)

For screening of hybridoma, the growing hybridomas are screened for their productions of antibodies by appropriate sensitive assay eg. Enzyme-linked immunosorbent assay (ELISA), Radioimmunoassay (RIA), Dot-blot (DB), Western blot (WB) and Immunohistochemistry (IHC) and the antibody producing hybridoma are cloned. High dilution of the cells are made so that individual cells can be transferred to tissue culture medium in microtitre plates and grown. Wells containing

one colony are then grown in culture. Hybridoma can be grown in tissue culture and up to 10 μg/ml of specific antibody may be obtained.

Advantages of monoclonal antibodies (Vetterlein et al., 1989)

(a) Each cloned B cell produces only one type of antibody of predefined specificity.

(b) Impure antigens can be used to generate monoclonal antibodies.

(c) MAbs can be generated against rare and weakly immunogenic antigen.

(d) MAbs have a predefined isotype and thus variable effector functions.

(e) Reproducible antibody can be made in unlimited amounts.

Several research was widely using VTG induction for evaluation of xenoestrogenic effects on various species such as common carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) (Nilsen et al., 1998). Although these fishes can live with polluted environments, fathead minnow, zebrafish, and Japanese medaka are all small and low degree in food cycle, not suitable to reflect the food chain accumulation effect and Japanese medaka's life cycle is about one year.

Monoclonal antibodie specific to VTG and ZRP in particular fish species from differrent regions which are not available in fish species from this region. Antibodie specific to VTG and ZRP from this regions has been developed for this purpose.

***Liza subviridis* (Valenciennes, 1836)**

Figure 2.5 *Liza subviridis* (Valenciennes, 1836)

The Greenback Mullet, *Liza subviridis* is an omnivorous fish feeding on plants, small animals and small crustaceans and other foods, and can reflect the food chain accumulation effect. The Greenback Mullet is found in the area from the Indo-Pacific. The habitant of this species is found in shallow coastal waters and enter lagoons, estuaries and fresh. The greenback mullet is economical the most important (Daham et al., 1991). The maximum size of this fish have been reported standard length 40 cm; commonly to 25 cm total length. The fish is heterosexual, exhibiting external fertilization. All the fish examined possessed gonads of a distinct sex. While maturing ovaries and testes can be macroscopically differentiated, gonads of immature fish can be sexually differentiated only microscopically. Externally, differentiation of the sexes is difficult, except in mature fish (Chan et al.,1980).

Table 2.1 Molecular mass of vitellogenin subunits reported in teleost fish.

Fish sepcies	Molecular mass (kDa)		Method	Reference
	Holo-Protein	Subunits		
Medaka (*Oryzias latipes*)	420	200	SDS-PAGE	Hamazaki et al., 1987
Goldfish (*Carassius auratus*)	380	147; 142	SDS-PAGE, Nondenaturing -PAGE	de Vlaming et al.,1980
Rainbow trout (*Salmo gairdneri*)	455	220	Gel filtration	Hara and Hirai, 1978
Tilapia (*Oreochromis niloticus*)	300	-	Nondenaturing -PAGE	Chan et al., 1991
Spotted Seatrout (*Cynoscion nebulosus*)	220	-	Gel filtration	Copeland et al., 1988
Landlocked Atlantic Salmon (*Salmo salar*)	520	176; 127	Gel filtration	So et al., 1985
English Sole (*Pleuronectes vetulus)*	330; 320	130	SDS-PAGE	Roubal et al., 1997
Grouper (*Epinephelus malabaricus*)	525; 260	140; 113	Nondenaturing -PAGE	Utarabhand et al., 1996
Gag (*Mycteroperca microlepis*)	439	183	SDS-PAGE	Heppell et al., 1999