Endocrine Disruption in Aquatic Invertebrates

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Good morning, Ladies and Gentlemen. Let me first thank the Ministry of Environment for giving me the possibility to be here.

This symposium has so far dealt predominantly with one phylum of organisms, the Phylum Cordata and the vertebrates. Today I want to say something about the effects of anthropogenic chemicals, especially endocrine disrupters, on the other 18 phyla of organisms, which comprise the invertebrates.

Before I really develop my talk, I want to just re-ask the question; why is endocrine disruption so special? I think we can come up with a few different answers.

Firstly, I think an assumption was made quite a while ago when the field of endocrine disruption was developing that endocrine disruption is special because it affects organisms at concentrations below other thresholds of toxicity. This is why the concern over endocrine disruption really grew so much.

The second thing is that we were concerned that endocrine disrupters affected processes that influence Darwinian Fitness. This will include things like metabolism, reproduction, viability of offspring, growth rate, etc.

One of the things we must consider when it comes to the invertebrates is; are these statements still true for invertebrates, or do other forms of toxicity interact with endocrine disruption — do they occur at very low concentrations, too? I hope that during the course of my talk, I will be able to draw attention to some of the possibilities in that regard.

Please do not think that these are in any particular order, but the point I was trying to make was that endocrine disruption seems to be occurring at lower exposure concentrations than perhaps metabolic toxicity, immunotoxicity and so on. So let us see if this is true in the invertebrates as well.

Let me say something about the invertebrates because I should imagine that most of this audience is not particularly familiar with them. Let us remember that 95% of the all the animal species on the planet are actually invertebrate animals. They come from a very wide range of phyla — there are 18 phyla listed here, things like sponges, sea anemones, coelenterates, corals with global distribution, various worms, mollusks, the arthropods, and in particular in the aquatic environment the crustacea, various worms, the annelids, and the echinoderms, the starfish.

Let us recall then that these organisms are the ones that populate all the ecosystems of the earth. They are responsible for structuring ecosystems and they are responsible for the ecosystem processes, the cycling of nutrients and energy, and so forth. They are hugely important, and if it were to be the case that our chemicals that we are releasing into the environment are not only toxic to them in the sense that we know already, but are also subtly perturbing their hormone systems, then clearly it will have a very important global significance.

The other thing to remember about the invertebrate phyla is that they have a very diverse biochemistry and physiology. Quite often they are completely different to the vertebrates. The role of hormones and different enzymes in the invertebrates is very different to the role of those same hormones and enzymes in the vertebrates.

In terms of their physiology, they are also extremely different. Some of them have gills; some of them do not have gills. Some of them do not have a gut that runs all the way through the body, they have a blind end gut. They are very different indeed. Many of the assumptions that we make, many of the things we know for vertebrates, are not true in the invertebrates.

I have just a few slides now to show you the diversity of forms. These are obviously clams that we are talking about; here is a crab from the crustacea. A sea slug, again from the mollusca. Note that how this mollusk is very different from the clams. Great difference in structure. Things like sea anemones… I could go on to show you a very wide variety of forms and structure in the invertebrates.

We heard yesterday when we were talking about the OECD and the testing systems that are available for protecting wildlife from the effects of anthropogenic chemicals from endocrine disrupters.

At present there are no test systems for protecting the invertebrates. There are many being developed, but generally speaking they include things like, for example, a shrimp assay for the crustacea. But clearly an assay that protects a crustacea may not be very helpful for predicting what will happen to something like an anemone. So we must bear this in mind when we are developing global strategies for protection of invertebrates from the effects of pollutants.

What are the key issues with regard to invertebrates? Is there evidence that disruption occurs in invertebrates in the first place? Well, we know that there is some evidence for some phyla, and I will mention the mollusks in particular in a moment.

Does it occur in all phyla? We do not know. And one of the reasons we do not know is we do not know what endpoint to look for in many of the different phyla. In sea anemones, for example, what would we look for as an endpoint to see if the hormone systems of that particular group are disturbed? We do not know yet.

Which are the most vulnerable phyla? Once again we tend to focus on the mollusks and the crustaceans because those have been traditionally used in pollution monitoring in the aquatic environment, but it may well be that the echinoderms or some other group are more vulnerable.

Most important of all, does endocrine disruption in the invertebrates lead to ecologically significant consequences? Does it alter population structure, community structure? Does it influence ecosystem processes?

Now I want to do a very brief review of endocrine disruption in the major groups, reviewing the bulk of the information that we have so far.

Endocrine disruption in the mollusks was brought to our attention first by studies on oysters and other bivalves exposed to organotin compounds, TBT. It was shown back in the late 70s and early 80s that oysters and bivalves exposed to these antifouling agents suffer reduced spatfall — they do not have so many successful larvae recruited to the population — and often they have abnormal development.

One of the reasons that we think that these mollusks are so susceptible to compounds like organotin is because they do not possess very high levels of the P450 detoxification enzymes, which would normally convert tributyl tin to dibutyl tin and monobutyl tin and then excrete it. In fact, you tend to get the accumulation of quite high levels of tributyl tin, so it can exert its effect. These detoxification enzymes are low. This may indicate that the mollusks are a particularly vulnerable group.

As I am sure many of you are aware, organotin, TBT exposure, results in imposex and intersex in a wide range of gastropod mollusks. That is a superimposition of male characteristics on female snails.

There is also a growing amount of anecdotal evidence from the literature, or at least from the gray literature, of ovotestis formation in a variety of clams and bivalves exposed to metals and metalloids, in particular arsenic, and a wide range of organic pollutants. But as yet definitive studies showing that this is an endocrine disrupter effect, I think are in an early stage. We need to do a great deal more work here.

This is just an example of an oyster, I am sorry the photograph is not very good. This is *Crassostrea*, a Portuguese oyster, a normal one. You can see the space where the oyster would normally live and a nice rugged shell. This is the same species of oyster exposed to organotin: a very small space for the animal to live and lamellation of the shell.

These studies were conducted in the early 80s. The significance of them is enormous on global scales, because around the world — this is actually in southern China — oysters (these are all oyster shells) are a major source of food for humans and of great economic importance. Clearly impacting these food supplies with endocrine disrupting antifouling agents is of major significance.

Imposex are the superimposition of male characteristics on female gastropods, particularly affects mesogastropods. If one surveys the literature, there are at least 100 species of gastropods that have been affected worldwide by exposure to organotin.

There was growing weight of evidence accumulating, and I think that many people now believe that the key reason for the imposex phenomenon is that testosterone accumulates in female snails because the enzyme that would convert testosterone to estrogen, aromatase, is inhibited by organotin and this leads to pseudopenis formation and so on.

That idea has been widely disseminated among people, but I think actually the real reason is something slightly different that was written by Gerry LeBlanc at North Carolina State University is really demonstrating that perhaps a more important mechanism than this, is this reduced esterification of testosterone in the female snails exposed to organotin which gives them higher free testosterone concentrations and effects a conversion.

We do know that in the case of these gastropod mollusks there have been significant declines worldwide due to the exposure to organotin.

These diagrams were some of the ones, which demonstrated the imposex phenomenon. Here is a normal male snail with the penis growing here and the vas deferens. This is a female snail exposed to organotin with a non-functional penis and the beginnings of vas deferens. Here is a late stage version, again a female snail, but the eggs have been trapped in the vulva by the growth over the vulva entrance, and so the eggs degenerate and the snail dies.

Moving on rapidly to crustaceans, barnacles have been shown to be affected in laboratory studies. In decapods, — that is crabs, lobsters, shrimps, and so on — there are reports of effects on moulting hormones, ecdysterone. There is some suggestion of ovotestis formation, and we know from Japan by the work of Dr. Takahashi that there is an intersex-like syndrome in freshwater crabs.

Potential effects on sex hormones seem to be possible, but we have looked at things like vitellogenin induction in crabs and shrimps and not found an effect; I will mention that a bit more in a moment. In northeast Britain, copepods — these small marine crustaceans, harpacticoid copepods — in heavily sewage polluted esteroids have been shown to have an intersex syndrome. And, developmental abnormalities have been seen in amhiropods.

I would make the point at this stage that many invertebrate organisms change sex throughout their lives; they change from being female to male, or male to female. Also, parasitism in many invertebrates leads to changes in sex.

So when we are seeing these phenomena, we must make absolutely sure that these are not natural changes, for example, the gonads of these organisms are in their various structures. We must make sure that it is not due to parasitism before we can really assume that anthropogenic chemicals are causing the problem.

This is another barnacle, and this is the first larval stage, the nauplius larvae of a barnacle, and this is the cyprid, the second larval stage of barnacles.

We have undertaken in my laboratory exposures of these barnacle larvae, which float free in the water and would normally settle on rocks and establish new colonies. We have done exposures with nonyl phenol at these kinds of concentrations, and have shown that the success of larval settlement is greatly reduced.

It was initially tempting to conclude that this might be something to do with the effects of nonyl phenol on larval development, it might be an endocrine disrupter effect; in fact, we now think that this is probably almost certainly due to the detergent properties of the nonyl phenol influencing the ability of the larvae to settle on rocks. So again, we have to take care.

However, we did do some studies where we examined a protein called cyprid major protein. This is an egg yolk protein analogous to vitellogenin, which is found in barnacle larvae during development. Again, we exposed groups of larvae to nonyl phenol at different concentrations, and we found in the first cyprid stage — these are the control values and these are the nonyl phenol exposed — that there is a significant increase in the level of cyprid major protein in the larvae at this stage, but by the time the larvae came to settle, the cyprid major protein levels were well below the controls, and these were statistically significantly different.

So we do seem to be exerting endocrine disrupter effects, but we do not know what the significance is.

We heard already about the importance of measuring vitellogenin in fish and what a wonderful biomarker it is potentially of exposure to estrogenic compounds. It turns out that crustacea have vitellogenin in their blood as well. So we undertook to see if crustaceans, particularly decapod crustaceans, exposed to endocrine disrupting chemicals, to estrogens, would also have an induction of vitellogenin.

We prepared extracts of ovarian tissue, we developed an ELISA technique — and this seems to be a very robust ELISA technique — and then we exposed decapod crustaceans to a very wide range of endocrine disrupters: nonyl phenol, bisphenol A, organotin and several other compounds including estradiol. Under no circumstances in the laboratory did we find any influence whatsoever on vitellogenin levels in male crabs, nor in female crabs. This once again emphasizes the differences that there are between the vertebrates and different phyla of the invertebrates.

I do not expect you to read this, this is simply the only abstract I have been able to find so far reporting oocytes in the testes of lobsters, another decapod crustacean. This is simply an abstract produced by these researchers, but I have never seen the follow up paper and as yet I have not found any other evidence of ovotestis formation in crustaceans, not widely anyway.

Moving on to work from Sweden, this is the work of Sundelin and Eriksson. These are normal embryos of amphipod, another small crustacean. If you collect these amphipods from the vicinity of pulp mill affluents along the Swedish coast, then you do find at rather low concentrations of the affluent that the eggs die within the amphipod, and that many of the embryos are highly abnormal compared with the previous slide that I showed you.

The authors of this particular work suggested that you could use these aberrant embryonic forms, these unusual embryos, these damaged embryos, as an indicator of endocrine disruption following exposure to pulp mill affluent. However, I think that we should view this kind of approach with caution since we do not have any direct evidence that this is endocrine disruption. It could be just normal metabolic toxicity, it could be genotoxicity, it could be all of those things combined.

This is another amphipod that has been exposed to nonyl phenol. This individual is a control and this is an exposed one. It is not a terribly good picture, but what I want to demonstrate is that on the nonyl phenol exposed male amphipod, that the antennae are greatly elongated compared with this one, which has gone off the bottom.

Now it is quite right that in male amphipods of this species, that the males at certain times in the reproductive cycle do grow these extra long antennae and they are secondary sexual characteristics, and the antennae are used in reproduction. But the interesting thing for us was that exposure to low concentrations to nonyl phenol induces extra long antennae in all the males in the exposed group very quickly. That might also be an endocrine disrupter effect.

That is the mollusca and the crustacea, which are representatives of the arthropoda. What about the other invertebrate phyla? Well, really we do not know and this is perhaps the most worrying thing of all. We do not really understand the hormone systems or endocrine systems of sponges, coelenterates, and all these other groups you can see here. And because we do not understand the endocrine systems, it is quite difficult to spot endocrine disruption. We do not know what endpoints to use.

We have been able to detect the presence of estrogen and testosterone and other steroid hormones in the tissues of representatives of these different phyla, but to establish a functional role for them is a long way off. We have not been able to do that in these groups. It has been suggested, in fact, that the presence of androgen and estrogen in the tissues may simply reflect the diet of these organisms. They may be contaminants from eating other organisms that do contain estrogen and testosterone. Clearly, further research is needed there.

In the echinoderms, however, the starfish and sea urchins and so on, there is evidence of functional vertebrate-like steroids. But as far as I am aware and there may be others who can tell me different, there is no evidence of endocrine disruption *in situ*.

To move into the latter part of my talk, is endocrine disruption confused with other forms of toxicity, and if it is, does it matter? Well, a few years ago if one found evidence — before the endocrine disruption became a subject of great interest — if we found mixtures of ovaries and testes, we might have concluded that this was a teratogenic effect, a developmental effect, perhaps due to damage to the genes by a chemical pollutant or to gene expression in some way.

Looking at the information that is available from one of the U.K. programs, Endocrine Disruption in the Marine Environment, it was found that in flounder that were collected from various polluted estuaries, some of them had vitellogenin induced, and in others they have the vitellogenin and over testes formation. But another group were found with no vitellogenin induction, but ovotestis formation.

One might suggest, then, that it is worth investigating whether these are different processes, and whether the ovotestis formation is a developmental teratogenic effect and that vitellogenin induction is perhaps an endocrine/hormonal effect. I do not know the answer to that, but I think we need to do work in that kind of area to answer those sorts of question.

Abnormal oocytes, larval development, reduced viability of offspring, and altered growth rates have all been identified in invertebrate organisms, and if you look through the literature you will see them reported as being effects of genotoxicities and metabolic toxicities. Quite often the genotoxicity of these things occurs at very low concentrations. So is this genotoxicity or endocrine disruption or what?

Let us remember when you talk about endocrine disruption that it is only one component in the factors that can affect the health of organisms. Endocrine disruption may be occurring simultaneously with neurotoxicity, genotoxicity, immunotoxicity, and metabolic toxicity. Furthermore, the biomarkers that we might use to measure endocrine disruption or any other phenomenon within the cell are intimately linked, are combined in all kind of ways.

For example, metallothionein that we heard about the other day will not work properly without the presence of glutathione. Glutathione levels are reduced by exposure to organic pollutants and so on. Stress proteins, the multi-drug resistant genes and multi-pollutant resistant genes. The stability of lysosomal membranes is influenced by stress proteins. All these things work in concert in the cell, and we should not forget then that endocrine disruption is just one of a whole suite of toxic mechanisms that proceeds in cells.

We have recently performed work on mussels, and have shown that there are pollution gradients in harbors in the USA that are reflected in changes in immunocompetence in *Mytilus edulis*. It is at extremely low concentrations.

I think that one of the things we need to do is to focus on detecting all different mechanisms of toxicity that occur in an organism using biomarkers and marine bioassays. Certainly I would say that we

are undergoing a transformation in the way that we approach toxicology. Chemical exposures, bioavailability, and chemical residues have been used in the past for toxicity tests to predict ecological damage.

Now I think we are moving much more to detecting biological effects *in situ*. We are using biomarkers to give us a clue to what kind of toxicity is taking place: it might be genotoxicity or endocrine disruption. Through this we then go on to look at the residues of chemicals in the tissues and make appropriate analyses. I think the process of measuring biological effects is changing.

We should also remember to put this into the global context because studies are going on worldwide. For example, the global ocean observing system, where we are not only looking at the impact of chemicals on biota, but also physical changes, changes in climate, and so on. We need to devise very sophisticated monitoring processes to detect all of these different toxicants.

Let me just finish now with the conclusions. Within the invertebrates, among the Mollusca, some species of gastropods and bivalves appear to be vulnerable to endocrine disrupters, especially associated with organotin exposure, and this leads to ecologically significant effects. On the basis of current evidence, I do not think there is very clear evidence that estrogen mimics are effecting mollusks in a very strong way.

Among crustaceans, laboratory studies indicate that they are not vulnerable to many estrogenic compounds that affect fish; that is in the laboratory at least. Specific pesticides we know do influence moulting hormones and some of the other hormones, and there is anecdotal evidence of ovotestis formation and abnormal mutagenesis. That might be due to endocrine disruption, but we cannot rule out the role of genotoxicity and so on.

For other invertebrate phyla, there is insufficient information. I believe that endocrine disruption must be considered in relation to other mechanisms of toxicity, and I hope we can have, at some stage, a lively debate about this.

Finally, I think overall monitoring programs to detect the impact of anthropogenic chemicals should be designed to detect many different kinds of toxicity and many different kinds of chemicals, and then we can use a weight of evidence approach to address the impact of these chemicals and evaluate their significance in the environment. Thank you very much.

Q&A

Iguchi: We have heard that it is hard to understand with only the example with invertebrates and endocrine disruption, and that various types of toxicity come into play and it is hard to sort them out.

We can take one or two questions. Does anybody have a question? Your name and affiliation please.

Q: My name is Hiroto Tamura from Meiji University. My major is pesticide science. In the field of pesticide science, endocrine disruption is a very important target to design highly selective pesticides between vertebrates and invertebrates. In your slide, the disruption of 20-hydrox-yecdysome is called the endocrine disruption in the insect. It is common sense in our field.

So how do we design effective pesticides? Because the former President Clinton gave award to the company so that the company developed a very nice insecticide which disrupts insect endocrine system to inhibit. That chemical is agonist of 20-hydrox-yecdysome. Please give me a good comment.

Depledge: It is a very difficult comment to answer. Of course, it is true that these chemicals are specifically designed to knock out insects and they will have an impact on crustaceans, but that has been true of a very wide variety of pesticides, synthesis of cholinesterase inhibitors and so on. It also affects arthropods in the aquatic environment. I think that we need to conduct more research to try and get more selective mechanisms.

I think in the global context there must be a tradeoff between trying to wipe out insects that cause disease and things like malaria or what you affect very large numbers of people and cause a great deal of human health damage and the likely impact of those chemicals running off into the environment at low concentrations and affecting things like shrimp farms, crustacean fisheries, crab fisheries, which in turn, if those fisheries collapse, especially in developing countries, will have a huge impact on human beings.

I do not think I know the answer to your question, but I think it is extremely important that you have raised it. It is a good point.

Q: Also I would like to tell you that inhibition of juvenile hormone also the target for…

Depledge: It is the same principle, yes.

Q: It is a good pesticide.

Iguchi: Please.

Q: George Daston, Procter & Gamble. Mike, I enjoyed your talk a lot. You put up at the latter part as perhaps a spark for debate the idea that endocrine disruption should be considered in the context of toxicology overall. I am not going to debate that. I am going to endorse it, and say that that concept also needs to be recognized and extended for vertebrate and mammalian toxicity. That is not to say that endocrine disruption is not an important mechanism. It is.

But it is one among many kinds of mechanisms of abnormal development, and sometimes we do ourselves a disservice by starting with assumptions about mechanism, first. I think really what we are doing is making observations, and then eventually understand mechanisms. I applaud your comments and hope we can consider not just the invertebrate talks but all of toxicology in that context.

Depledge: Thank you.