UpClose

with Maria Sepulveda

From her lab at Purdue University, Dr. Maria (Marisol) Sepulveda has spent the last decade studying the impacts of environmental contaminants on fish and other wildlife, particularly on reproduction and early development. As a toxicologist with a background in veterinary medicine, her research also extends to parasitic diseases and the role they play in the health of wildlife populations. Together with her research team, Dr. Sepulveda is currently investigating the effects of pharmaceutical chemicals found in Lake Michigan on several organisms at the bottom of the food chain. She is joined by Dr. Cecon Mahapatra, a research scientist specializing in the genes involved in toxicity, and Chris Klinkhamer, a Masters student with a background in engineering. The study, funded by Illinois-Indiana Sea Grant, is among the first to collect data on two common contaminants in Lake Michigan and takes an important step towards securing the long-term health of the lake.

Just a few months before the study is scheduled to be completed, IIISG sat down with Dr. Sepulveda and her team to learn about the results so far, what they mean for wildlife in Lake Michigan, and the complex, and sometimes tricky, process of conducting a year-long study on live organisms.

Where are you in the project now?

Cecon: We got the funding in April, and by May we were trying to grow all the organisms. We started with *Daphnia* [a small planktonic crustacean]. We got the algae from a place in Texas, and the diatoms [a common type of phytoplankton] we ordered from outside. But they didn't survive well, so we had to get them from a different lab.

Maria: We have developed all the protocols to grow the organisms and to keep them in good condition throughout the experiment, and we have tested the two pharmaceuticals that we said we would test—cotinine and triclocarban—in diatoms and algae. We have done a lot with *Daphnia* as well, but the survival was not that good. So, we are repeating it.

Cecon: With a fresh *Daphnia* colony. They are ready to go now.

Maria: The fish [fathead minnows] will be the last species we test.

Was the survival of *Daphnia* low because of the pharmaceuticals?

Maria: No. Every time you start working with something new, it takes a while to get your protocols. You can't run a test if you don't have at least 85 percent survival in the control group. If you have less than that, you have a problem in the set up. It could be the wrong temperature; it could be anything.

Cecon: We were getting the *Daphnia* from a lab at Purdue. They grew it for their own experiments. Maybe the colony was getting old or something to do with that. They also had the same issues. When we got them and grew them in our incubator, they were not reproducing the way they should.

Is this a common thing to happen in studies like this?

Maria: Yes. Unfortunately, yes. You can have a colony that is reproducing great, producing hundreds of eggs a day, and then, when you want to start your experiment, they stop laying eggs, and you try to figure out what happened. It is always like that.

Cecon: Then you pray. That is the only thing we can do.

When something like that happens, do you have to start over from scratch?

Maria: Yes. In the case of the *Daphnia,* it is relatively easy to start from scratch because they grow relatively fast. They have a very short life cycle. In the case of the fish, it takes fathead minnows about 5 months to grow to adults and lay eggs. So, if you lose your adult population, it takes about 5 months to get a new population. We always keep a population of juvenile fish to work with as a backup, so we don't have to wait half a year to start.

But the environmental walk-in chamber that we use was not working properly. The temperature is fluctuating. We had to make a new system, so we had to take all the fish out. They didn't like it when we did that, and they stopped laying eggs. Now we are waiting and trying to figure out when they are going to lay again.

Cecon: It is just a reaction period because we changed the facility.

Maria: But this is typical when you work with live animals.

But you have finished testing the impacts of cotinine and triclocarban on some species, correct?

Cecon: Yes, we have finished. There is no data on diatoms, so this is the first time we will get some data for the diatoms. For the algae, I think there was one study.

Maria: We chose those two from the study, which was not even published when we looked at it, that was funded by Illinois-Indiana Sea Grant http://www.iiseagrant.org/research/reports/bernotlauer_finalreport_20jun11.pdf. They had received funding to do a preliminary assessment of the pharmaceuticals found in Lake Michigan. They published that report right around the time we were writing this proposal. We selected the chemicals based on that list, but, since the amount of funding was very small, we were limited to selecting only a couple to study. We chose those two because there is almost no data in the literature on their effects on aquatic organisms.

Triclocarban is a metabolite of triclosan, which is an anti-bacterial chemical that is in toothpaste, soaps, detergents—all kinds of household items.

Cecon: And cotinine is a metabolite of nicotine. You can test the level of cotinine in the blood and say whether someone is a mild smoker. Even people who are exposed to second-hand smoke will have cotinine in them.

The initial results really look promising in some cases. We took the concentrations that are reported in Bernot and Lauer as the maximum. Now we have an idea, and we are testing more concentrations and going beyond that level to see the range of impacts.

What impacts are you seeing from these chemicals?

Maria: For the algae, what we do is look at growth inhibition. We are not looking at how many die, so it is a little different than when you are looking at fish. For the algae and diatoms, we are talking about percent of growth inhibition. Like Cecon was saying, we are trying to test a wide range that includes what was found in Lake Michigan but is above and below that. Then we can calculate the concentration at which 50 percent of the animals stop growing, which is called the EC_{50}. We can't compare these results to any other study, because there aren't any, but this is what we are getting: the concentration in Lake Michigan is right around the concentration that we are seeing having an impact on growth [in the lab]. This would suggest that the concentrations that are out there now are harmful to these organisms.

Is that the case for both of the chemicals you are testing?

Maria: No. That is for cotinine. For triclocarban, on the other hand, we don't see much. It doesn't appear to be causing any issues at the current level.

Cecon: At the Lake Michigan concentration, we don't see a conspicuous change in the growth. That is why we are going on to predict what concentration would be harmful.

Is it surprising that you don't see effects from triclocarban at the current level?

Cecon: You know, we see what we see.

Maria: The numbers are going to vary between different species and different studies. The values we are getting are hard to compare because there is just not a lot of data on triclocarban. The table we created for our proposal [that lists the chemicals being tested and their known toxicity] has only one EC_{50} number that someone else has reported for algae: .0001 milligrams per liter. That is a different species than we are working with. There is a lot more known about triclosan. But triclocarban is very prevalent because it is what triclosan becomes in the environment. It is interesting that there is not a lot of data. The only data we could find was from an EPA report, not from a published, peer-reviewed journal.

How do you predict the level at which the chemicals would be harmful?

Cecon: We are increasing the concentrations. We repeat the same experiment exactly, just with higher concentrations. We are going to double what is in Lake Michigan, times it by four, then by eight—until we have the concentration where they die.

Why test at higher concentrations than what is in the lake?

Cecon: It gives us an upper bound. It tells us at what concentration growth is inhibited. The current concentration in Lake Michigan might not be harmful, but it could go beyond that level.

Maria: And, for us to calculate toxicity, we have to have mortality or growth inhibition. If we just stayed at the current concentration, we would never be able to calculate an EC_{50} value.

And what does an EC_{50} value tell you?

Maria: That is the effective concentration. You can have an EC value on reproduction or growth—it is not mortality, but an effect you are measuring.

For the fish, we are testing mortality, LC_{50} (lethal concentration). We will test the hatchability and survival of the fish at different concentrations. I wish we had the funding to do more measurements. Mortality is really the minimum

you can do. When you don't know anything about a chemical, that is the first thing you look at: survival. But then you want to do more. You want to look at sub-lethal effects. For example, why are the fish not hatching, or why are they not growing. But all we can do right now is report that this is the concentration that kills 50 percent of the embryos.

Are the results from short-term or long-term exposure?

Cecon: Chronic [long-term]. This is from a seven-day exposure.

How does the short-term test differ from the long-term test?

Cecon: It is in our experimental design that we will do a 48-hour acute test [short-term exposure].

Maria: Looking at these [long-term exposure] numbers, we might not see much with a short exposure time. If after seven days there is nothing going on, it is going to be even less at 48 hours. For cotinine, the effects could start after 48 hours, but we will have to look at that.

What would it mean for the Lake Michigan environment if there wasn't an acute reaction for either of these?

Maria: It wouldn't really mean a lot. Like I was saying, these values are just a first attempt to figure out what the chemicals can do. Then, once you figure out what the EC_{50} value is, whether it is acute or chronic, then you have to be able to move beyond that. What is the mechanism of this chemical? Why are the diatoms responding this way? And then you have to go back to the field, to Lake Michigan, and do a much more thorough assessment of what is in the water. Right now, we can report a value that might be off of what is out there. We don't have a lot of information about that now.

Is it accurate, then, to say that this study is just the first step in determining the impact of these chemicals?

Cecon: Definitely.

Maria: And we are only doing [a detailed examination of] two chemicals; there are at least nine more [found in the lake]. In one of our studies, we are attempting to look at these chemicals as a mixture, so we do have some data on that. We have exposed these organisms to all of them together, at the concentrations reported in the Bernot and Lauer study.

Cecon: In the environment, organisms are usually exposed to a mixture. They are not exposed to just one chemical.

Have those tests been completed as well?

Cecon: We have completed the test with algae. We have tested the concentration in Lake Michigan, half of that, a fourth, an eighth, and a sixteenth. I haven't calculated the EC_{50} yet.

Maria: But just eyeballing these results, it is looking like the mixture is causing a significant decrease in the growth of the algae. We would like to repeat everything at least twice to make sure that the numbers are repeatable.

Are you testing just the proportions of each chemical found in Lake Michigan?

Cecon: We just took the maximum value of each chemical reported in Lake Michigan and put it in one solution.

For the diatoms and algae, are you looking at affects other than growth inhibition?

Cecon: Not in this study. We are just looking at the growth. But with the *Daphnia,* we are looking at reproductive rate and survival.

What do you expect to see in the *Daphnia* at these concentrations?

Maria: It is difficult for us to compare both of those chemicals because of the limited amount of information that is out there. If we base it on triclosan, which is the parent compound [of triclocarban], just to get some idea, it doesn't seem to be that toxic. But, again, we are not sure if the metabolite is the same.

Chris: It is just one of those things that you don't know until you do it.

When do you think you will complete the tests on the *Daphnia*?

Cecon: We are expecting to finish at least within eight weeks. With the fish, I don't know if we will finish in the eight weeks. It might take a little bit more time because the fish are not laying eggs, so we have to wait for that. But we do have the egg-laying population.

Can you tell me a little about what you do day-to-day for a study this large?

Chris: For the algae and the diatoms, it is pretty easy. You just start the experiment, and it runs itself. You wait for seven days, and they will grow. All you have to do is shake them once a day so that it [the water] is aerated and they are not stacking on top of each other. I am giving the algae a few days for the cell count to increase before starting the next study.

With the *Daphnia*, I change the water every day. I have about 180 little individual *Daphnia* cultures going. Every day, I change each of those, put in new chemicals, feed them, and record if they have hatched any eggs. I will store the new hatchlings so that we can do genetic analysis on them—and just repeat every day for seven to eight days. As long as I have about 30-40 females that are adult age, it is good.

Photo courtesy of Christopher Klinkhamer

That is the number needed to start the study?

Chris: Yes. The EPA has certain guidelines. They need to be laying at a certain rate, and with a certain number of offspring, before you start the study so that you know they are healthy.

Maria: And there are all of these other parameters that you have to measure, like the temperature, and control at certain levels the whole time. Otherwise, the study is not standardized.

Cecon: We are following the EPA protocol for all these experiments. If they are healthy, then we know that they don't have problems from any other stressors. It is to ensure that they are exposed to only this stressor [the pharmaceuticals]. And, also, the protocols work for us. The incubator we had initially had some fluctuation in the temperature. It was showing some problems, and we had a growth rate problem. The *Daphnia* were not growing well then.

Maria: In Lake Michigan, of course, temperatures are going to vary widely depending on the depth, on the time of the day—all kinds of things. Here, it is all constant. It is one temperature for the whole study.

How much connection is there between the lab and the real-world, where characteristics like temperatures, pH levels, and dissolved oxygen differ?

Maria: What we are seeing in the lab is not necessarily transferable to what is going on in the environment because the temperature is going to drive everything. If you have low temperatures, everything is going to go slower, and you might not have a lot of effects because of that. But, if you have higher temperatures, you might see very different effects.

If it is not directly transferable, what do these results tell us about the health of Lake Michigan?

Maria: It is a very initial starting point. Like I was saying earlier, if you don't know anything about a chemical, the first thing you do is test the LC_{50} or EC_{50}, and then you go from there. But there are many more things you need to do in order to determine if there is risk or not in the environment. If I say the EC_{50} is a certain level, and the lake has the same concentration, I could hypothesize that that is a problematic concentration. But, unless this is supported by long-term studies that measure the actual levels that are in the lake and measure the actual responses of the organisms that you are measuring in your lab out in the environment as well—a lot of things need to fall in place and happen. This is just the tip of the iceberg.

Cecon: The thing you are asking about the temperature—that is a completely different study, and people will do that study to see the effects at different temperatures.

Are your results conclusive enough to help natural resource managers know what to look for when monitoring concentrations in the lake?

Maria: I think more data needs to be collected. The information that we have is very limited and preliminary. We can't say that those are real levels. They might be, but they might not be. I would think it was a very small area that they [Bernot and Lauer] sampled.

Cecon: At least this study will give you information on how the organisms may behave, where as we don't have that data right now at all. You will get an overall perspective.

Chris, you mentioned that you are waiting for the algae's cell count to increase before starting the next test. Why did you decide to do that?

Chris: We are going to start one of the algae experiments, and we start them at 10,000 cells per milliliter. The mass culture wasn't growing quite as fast as I wanted. We are expanding the range of the concentrations we are exposing them to. So, just to make sure I didn't run out half way through, I waited an extra day. They will reproduce and be at a higher cell count today. You start the experiment when they are at their maximum growth rate, which is 4-7 days after you inoculate the growing medium. Today will be five days since I inoculated them, so they should be about at their maximum growth rate right now.

From the impacts you have seen, what bioaccumulation might there be?

Maria: I wouldn't say that there is a high risk of bioaccumulation. But diatoms are the base of the food chain in the Great Lakes. Fish are going to depend on the survival of diatoms. If the population of diatoms is not healthy, you can have repercussions higher in the trophic level. Not because of direct effects of the chemicals on other animals, but indirectly because there is less food. These chemicals are very different than pesticides, dioxins, or even heavy metals. They don't stay in the environment very long. They have a relatively short half-life. They tend not to bio-accumulate or bio-magnify because of that.

Is that the case with pharmaceuticals in general?

Maria: Yes. There are some exceptions. Some tend to accumulate because they are more lipophilic. But I would say that these are not very likely to go up the food chain like that. Although, again, there is really very little data. I have not seen many studies where they have measured these chemicals in predatory fish tissues, for example.

How do you plan on communicating results to the public once the study is completed?

Maria: There are different ways. In West Lafayette, there are always opportunities to communicate this type of information to the lay public. For example, they sometimes call me to talk at *Wednesdays in the Wild*. I have been there two or three times, and I have talked about pharmaceuticals there—I didn't have this data at the time. Those are avenues that we can

explore, but there are also things that we may not know of. We don't do outreach on a regular basis, but that doesn't mean we wouldn't want to participate in regular activities.

And, now, all graduate students have to come up with an outreach project, which is one credit. They are required to come up with something where they show what they have done, like go out to the schools and talk to the kids. There is another student in the lab that is doing pharmaceutical work too, and her project is on that. I think she is going to come up with a pamphlet on the issue of pharmaceuticals. That will tie in very well with this project.

Do people seem interested in work like this when you go to these outreach locations?

Maria: Yes, they are very interested. The people who show up are there because they really want to learn about the issues.

Chris: I would say Lafayette in general is pretty interested in science. There are a lot of bars and coffee shops where scientists will come and talk, and they get pretty good turnouts.

Maria: The newspapers are also always reporting about the projects that are going on.

Cecon: At a personal level, I have stopped throwing my pharmaceuticals in the dustbin.

Maria: That reminds me—about three years ago, there was nothing here on how to properly dispose of pharmaceuticals. There was nowhere you could take them, except maybe a police station. There are several [locations] now. And, three years ago, I participated in an event where we went to the farmers market. We had a booth and told people to bring their unused medicines. We collected around 300 lbs. in less than two hours. That barrel was full.

Are the collection programs permanent?

Maria: Yes, now there are permanent ones. There are at least two that are permanent now. And, now, there are all these things going on with a group called Go Greener. They do regular pick up events. They are very successful.

A police officer in Lafayette started one on her own—said this was something she wanted to do for the community. It is really taking off. It is open only once a week, but a lot of people know about it and drop off their medicines. Like I said, there was nothing four years ago. And I want to think this is in part because of what we have done—going out and talking to people and giving these talks. That could have helped some.

How did you three become interested in issues of pharmaceutical contamination?

Maria: For me, it's because it has been an issue for a long time. I see it as an important problem that needs to be addressed. There is a lot more data now than there was five or 10 years ago, but there are still a lot of things that are unknowns. I think it is something that needs to be done. It is interesting.

Also, when you talk to people about it, they are interested because it opens their eyes. They can't believe that this is going on, and it makes them rethink the way they live. It's related to us.

Cecon: We are responsible.

Maria: We are 100 percent responsible, right. These are the things we take, medicines we take on a daily basis that we are putting out into the environment. Everyone is responsible to some degree, and I think that is why people take it seriously. It is their responsibility to deal with it.

Cecon: Previously, I was working on only heavy metals. Then, after coming to Marisol's lab, I am working on different projects. We wrote this project because not much has been done. Marisol did a project on pharmaceuticals in the Wabash River, but we need more data in this area. And it is similar to the kind of work we are doing with the other chemicals [in other studies]. We are going much beyond preliminary work with the other chemicals. But here we are in the preliminary stage. The preliminary data looks good. Once we collect all our data, we really want to go more into how it affects organisms.

Chris: I come from more of an engineering background. I have actually taken wastewater treatment design courses. You talk about these emerging contaminants, but there is no real regulation for them. When you design a wastewater treatment plant, you don't design to take these kinds of contaminants out, and it is because this type of study hasn't been done. We don't really know what the pharmaceuticals are doing.

Studies like this could influence wastewater treatment work?

Maria: Yes. There is already a lot of research going on in that field. People are looking at how to improve the treatment facilities so that these things don't come out in the water. But, it is one thing to do that and another for the plants to upgrade, because they aren't required to measure any of this. If they are not required to measure any of this, why would they change when they would have to put a huge amount of money into their plants to upgrade them? The technology is here in many cases, but it is just too expensive. There are no regulations for these pharmaceuticals. Plants are only obligated to measure, if I remember right, heavy metals like mercury and arsenic. They are also obligated to measure *E. coli* and then just basic water parameters like turbidity and dissolved oxygen. That is it.

Work like this can also provide the groundwork for future regulations, then?

Cecon: Definitely. And maybe we will see things like small containers given by West Lafayette Cleaning, our trash collector, where people can dispose of pharmaceuticals.

Maria: We have been waiting for years, and we are going to continue to wait. Some states are taking their own measures, like California. For example, with EE2, which is one of the most studied pharmaceuticals and is found in birth control pills, California is discussing regulations for that way ahead of EPA. Some states are moving faster and are starting to regulate those things. But that is only one out of many, many pharmaceuticals.