Proposal to:

Interagency Ecological Program
California Department of Fish and Game
4001 N. Wilson Way
Stockton, CA 95205

Submitting Organization:

The Regents of the University of California
Office of Research, Sponsored Programs
1850 Research Park Drive, Suite 300
University of California
Davis, California 95618

Title of Proposed Research:

What would fish be without food? – A long-term effect assessment of pesticide mixtures on aquatic invertebrate communities using mesocosms

Total Amount Requested:	**Proposed Duration:**	**Desired Starting Date:**
$66,459	01/01/2013 – 12/31/2013	01/01/2013
Principal Investigator:	**Department:**	**Phone Number:**
Richard Connon	VM: APC	530-752-3141

Checks Made Payable to:

The Regents of the University of California

Send Checks to:

Cashier's Office
University of California Davis
PO BOX 989062
West Sacramento, California 95798-9062

Send Award Notice to:

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1850 Research Park Drive
University of California
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Approvals:

[signature] 08.20.12
Principal Investigator — Date

[signature] 8/21/12
Department Chair — Date

Dean, College/School (if required) — Date

Other Endorsement (optional) — Date

[signature] 9-6-12
Official Signing for Organization — Date

Erlita Oliver
Contracts and Grants Analyst, Sponsored Programs

I. Name of the Principal Investigator with affiliation:

Dr. Richard E. Connon - School of Veterinary Medicine, University of California, Davis.

II. Name of Co-Principal Investigator(s), with affiliation(s) and role in proposed project:

Dr. Sharon P. Lawler - Department of Entomology, University of California, Davis.

Role: Dr. Lawler has more than 20 years of experience in field studies and aquatic community ecology. The proposed methodologies are of common use in her laboratory and all the equipment needed to conduct this project is readily available. She enriches the proposed study with her extensive knowledge on aquatic community ecology, food web effects and pesticide management. As such, Dr. Lawler will be involved as advisor and student co-supervisor, assisting with taxonomy and community classification, and the preparation of reports and publications.

III. Project title:

What would fish be without food? – A long-term effect assessment of pesticide mixtures on aquatic invertebrate communities using mesocosms.

IV. Total budget:

We request a total of $43,155.31 plus overheads (54% for Federal Funds)

V. Study duration:

Funding is requested for a one-year period (from January to December 2013). A mesocosm system will be installed before the beginning of the project (November 2012) to allow the communities in the tanks to stabilize. Baseline measurements will be taken weekly during the month prior to pesticide application in March. Weekly sampling will be conducted until October 2013. Data collection and analysis will be continual and cumulative, and will be fully analyzed in the last two months of the project.

VI. Priority research topic and questions addressed and overall relevance:

Our proposed research directly addresses priority research "*Topic 3: Food web effects on fish populations (bottom-up effects)*", and investigates how seasonal invertebrate abundance and distribution are affected by interacting dynamic and stationary habitat components, and specifically the effects of pesticide mixtures in the ecosystem.

This project will generate new long-term information on the impact of chemical mixtures on aquatic invertebrate communities and their interactions as important components of food web dynamics in the Delta, from a bottom-up approach. Such interactions are not detectable by conventional laboratory toxicity tests, and can be difficult to discern in large-scale, low replication field studies. In addition, valuable data will be obtained on chemical fate, which is directly linked to ecological availability and toxicity. The information gathered will be a valuable resource for future watershed management and help protect the fragile biocenosis of the Delta. The study will result in at least one peer-reviewed publication in a journal of significant impact; e.g. Environmental Science and Technology or other scientific journals with similar impact factors.

VII. Overall project purpose:

The overall **purpose** of this project is to investigate the effects of contaminant mixtures upon aquatic invertebrates of ecological importance as food sources for fishes in the Sacramento-San Joaquin River Delta. We used a literature review to choose several widespread contaminants in the mixture to maximize the applicability of the study. These are a type I pyrethroid, permethrin, a type II pyrethroid, lambda-cyhalothrin and an organophosphate, chlorpyrifos. We will develop and use a controlled mesocosm-system for a realistic, yet replicated and controlled study.

Amongst one of the most pressing needs in the field of ecotoxicology is a better understanding of the effects of pesticide mixtures in the environment. Proper consideration of contaminant mixtures is of extreme importance, as their combined action can result in effects that are different to the effect expected from each compound individually (Lydy *et al.* 2004). Several studies have examined the effects on chemical mixtures on single aquatic organisms (non-target species) (Bailey *et al.* 1997; Anderson and Lydy 2002; Jin-Clark *et al.* 2002; Denton *et al.* 2003; van Wijngaarden *et al.* 2004; Brander *et al.* 2009). They can act synergistically, additively or in an antagonistic manner dependent not only on their modes of action, but also on their interaction with environmental variables, and of course species sensitivity. However, no studies have examined the mixture effects of the pesticides used in this study on aquatic communities and ecosystems, even though both synergistic and indirect effects are likely from pesticide mixtures (Sibley *et al.* 2000; Relyea 2009).

Our **goals** are to conduct a detailed assessment of the impact of two pyrethroids, lambda-cyhalothrin and permethrin, and the organophosphate, chlorpyrifos, on invertebrate communities, both macroinvertebrates and zooplankton, including monitoring the chemical fate of the three chemicals in both the water column and sediment.

Our specific **objectives** are thus:

1. To determine long-term contaminant mixture effects on the community structure, function, and biomass available to fishes, encompassing different life stages of aquatic invertebrates and their seasonal development.
2. To monitor the fate of contaminants, both in the water column and sediment, and how this passage affects the species living in the different habitats.

We hypothesize that contaminant mixtures will significantly affect the biomass, structure and function of the invertebrate community. Our work will quantify the degree of impact to invertebrate resources plus the timeline and trajectory of recovery.

The proposed study will address and answer numerous significant **questions** including:

a. How will pesticide mixture exposures affect the biomass and composition of invertebrate communities?
b. Will systems recover and if so what is the timeline?
   Will a certain species be replaced by another, less sensitive one? And if so, how does this affect prey availability and predator dynamics? How long will the pesticides be detectable? Do pesticide effects persist after levels become undetectable?

VIII. Project background and conceptual model:

Background: Disturbances of aquatic ecosystems by pollutants such as pesticides are considered one of the major threats to freshwater biodiversity (Dudgeon *et al.* 2006). Aquatic ecosystems and food webs in the Sacramento-San Joaquin (SSJ) River Delta are frequently exposed to areas of intense pesticide use that discharge complex mixtures of contaminants into surface waters. Due to the high use of chemicals, this area has increased levels of pesticides detected in water, sediment, and biota (Werner *et al.* 2002; Weston *et al.* 2004; Amweg *et al.* 2005; Werner *et al.* 2010; Weston and Lydy 2010). The occurrence of insecticides such as organophosphates and pyrethroids is of particular importance due to their broad-spectrum aquatic toxicities (Amweg *et al.* 2005; Bacey *et al.* 2005). In a recent study pyrethroids were detected in 52% of the water samples collected along the American River in the Central Valley (Weston and Lydy 2012) and a survey study within the San Joaquin watershed showed the presence of organophosphates at levels frequently above water quality standards (Bailey *et al.* 2000). A study monitoring acute and chronic water column toxicity in the Northern SSJ Estuary using the amphipod *Hyalella azteca* detected acute toxicity most frequently during winter and early spring of 2007 (Werner *et al.* 2010). At this time of the year endangered delta smelt are spawning and rearing in this area (Bennett 2005), and are dependent on a food source of small zooplankton, most importantly copepod species (Nobriga 2002); several others including sport fishes also spawn at this time (Moyle and Cech 2004).

The toxic effects of contaminants on aquatic ecosystems are often subtle and can be challenging to detect and quantify, especially in low concentrations acting as mixtures. Contaminants can result in reduced ecological fitness, and consequently impact differently on survival of individual species at different trophic levels, through sublethal physiological, behavioral, or immunological effects (Scholz *et al.* 2000; Medina *et al.* 2002; Lee *et al.* 2006; Connon *et al.* 2011), potentially leading to changes in food web and ecosystem dynamics (Werner *et al.* 2010). Research on contaminant mixtures has primarily been conducted as laboratory studies. Very few studies have investigated the effects of mixtures on multiple levels of biological organization and even fewer include chemical fate (Cuppen *et al.* 2002; Van den Brink *et al.* 2002).

Current risk-evaluation procedures generally evaluate effects on individual pesticides; i.e. through single compound exposures, and do not take into account the effects of toxicants at different levels of biological organization. They typically do not include a variety of interacting trophic levels within aquatic ecosystems. However, it is common within agricultural practices for several pesticides to be applied simultaneously and repeatedly, with differing combinations over time. However, the protection goals of legislation and regulatory authorities include populations, communities, and ecosystems, in addition to individuals. Thus more focused studies are necessary to ensure that toxicological assessments are more fundamental and effective, towards aiding ecosystem management efforts.

A mesocosm study is a highly suitable approach that can be used to assess simultaneous effects at the individual and community level. Mesocosms are useful surrogates for important cause/effect pathways in natural systems (Odum 1984). Mesocosms are established by collecting organisms and sediment and placing them in appropriate tanks or artificial ponds. The test systems hence contain naturally developed aquatic communities and naturalized sediments. A major advantage of such model ecosystems is that they simulate realistic exposures of interactive trophic levels of aquatic organisms to pesticide mixtures. Both direct and indirect effects on potential recovery of a wide array of species can be studied while allowing for interactions between the various populations within a community (Carpenter *et al.* 1985). Moreover, from a chemical fate perspective, dissipation and accumulation of a chemical dose can be observed under realistic conditions (Giddings *et al.* 2002)

Our **conceptual model** is presented graphically in Figure 1 (see appendix). Mesocosm systems combine a relatively high degree of biological complexity with low levels of replicate variability, and have the advantage of being easily constructed and maintained. Basic information on resource requirements of all key species within the aquatic community is important for the construction of a food web model as a tool to assess the ecological risks of an insecticide. The invertebrate community reacts to a series of indirect effects that result in ecosystem changes following, and in turn resulting from, toxic effects. For example, a reduction or elimination of pesticide-susceptible species has been shown to result in a disturbance of the trophic interactions within a community (Brock *et al.* 1992).

Direct effects of pesticides at the environmentally relevant levels used in this study are likely to be less severe than indirect food web effects. Fish species depend on a diet composed of various insect and invertebrate species, either in their early life stages only or all lifelong. For example, Delta smelts feed primarily on plankton typically found in places with lower velocities, such as copepods, cladoceran, amphipods and insect larva. Thus, it is our intention to investigate how the total biomass of fish prey is affected and whether preferred prey is affected (positively or negatively). We will also investigate how predator-prey interactions within the invertebrate community will be affected. For example, potential high mortalities of top-predators ultimately will lead to less predation pressure on prey species. Their absence allows some taxa to gain in abundance until the number of the top-predators recovers. Recovery is achieved when the abundance is in line with control abundance. Planktonic algae will not be identified in the proposed study but the photosynthetic activity will affect parameters such dissolved oxygen and pH. We will utilize turbidity as well as chlorophyll A concentrations as a measure of primary production.

The range of ecotoxicological effects in an ecosystem is not restricted to death or survival of species. The interactions between biotic and abiotic elements and the diversity of species and living spaces lead to a far greater complexity. In addition, aquatic organisms can vary broadly in their reproductive and dispersal ability. Sensitivity to toxicants is known to vary between species, but sensitivity can further vary between the different development stages of an organism (Williams *et al.* 1986; McCahon and Pascoe 1988). There are also instances where older organisms have been shown to be more sensitive to a particular toxicant than younger ones. For example, studies with daphnids and chlorpyrifos have indicated that older organisms are more sensitive than neonates to chlorpyrifos (Naddy *et al.* 2000). One possible reason for this is that the older animals have an increased filtration rate and receive a higher dose of the chemical, as pesticides bind to algae, thus uptake is directly associated with food consumption. By understanding both the significance of different routes of uptake for different classes of chemical and the physiology of an organism, it may be possible to predict differences in species sensitivity and hence interpret effects according to life stage (Boxall *et al.* 2002).

IX. Estimated number of all FESA and ESA-listed fishes that would be captured by the field component of your study:

N/A. No fish will be used in the proposed studies.

X. Project description:

The objectives of our proposal will be achieved in a single task which is designed to answer the specific research questions.

1. **Task 1:**
   Assessing the effects of pesticide mixtures on invertebrate communities

2. **Investigator(s) responsible for carrying out the task:**
   Drs. Connon and Lawler, graduate student Simone Hasenbein, along with direct assistance from a junior specialist, and an undergraduate student.

3. **Specific questions the task seeks to address:**

The overall questions this research seeks to address can be summarized into:

1) How do pesticide mixtures affect community structure?
2) What is the transport and fate of the pesticide mixtures within the water column and sediment?

4. Approach and methods:

Pesticide mixture effects on community structure: The mesocosm-system used for this study will be an outdoor-system at the UC Davis Putah Creek Riparian Reserve consisting of 17 solitary PVC tanks. Each tank will be filled with a layer of a clean sand-organic compost mixture. The mixture will mostly consist of sand to avoid eutrophication of tank water. To fill the tanks with approximately 350 gallons of water certified clean water and uncontaminated pond water from a pond in close vicinity to the study site will be used. No pesticides are in use near this pond, so contamination can be eliminated. Upon water use this will be confirmed by conducting a 96h toxicity test using the sensitive amphipod *Hyalella azteca* as described elsewhere (Werner *et al.* 2010). Aquatic flora will be obtained from the same uncontaminated pond.
When the mesocosms are set up, functional groups of invertebrates relevant to the fish species in the Delta will be in the systems; including but not limited to copepods, cladoceran, amphipods, and chironomids. Immigration of flying aquatic insects from Putah Creek and adjacent ponds will promote the development of an intact community, and supporting recovery by recolonization following pesticide application, thus strengthening the ecological relevance of this study. Flightless Taxa with slow reproduction such as amphipods will be added to respective tanks if loss is prolonged.

Pesticide treatments and controls will be randomly assigned to the tanks. To model realistic application scenarios pesticide formulations will be used for this study. As shown in our lab formulations can be more toxic than pure compounds (Beggel *et al.* 2010). Pesticide concentrations will be based on known environmental concentrations and low-level concentrations known to result in sublethal effects (Hasenbein, unpublished data).

Sampling of invertebrates will take place weekly from 4 weeks before pesticide application and eight months past application. Each sample will proceed as follows. For zooplankton identification we will collect one sample consisting of two sub-samples per mesocosm tank. By using a stainless steel tube (4.8cm in diameter and 1m in length) one sub-sample will be taken from the close vicinity of plants and another one from the open water. By noting the depth of the sampled water column the total water volume taken can be calculated. Water will be poured through a stainless steel sieve (pore size = 63µm) to remove metazoans and zooplankton will be collected from both sub-samples. Remaining water of the sample will be poured back to the corresponding tank. Animals in the sieve will be transferred to polyethylene-bottles and fixed with staining solution. For macroinvertebrate identification there will be two samples per tank, one taken from the water column using a sampling mesh and one from the sediment using purpose-built artificial sediments. To collect emerging invertebrates floating emergence traps will be positioned in each tank and sampled weekly. At the top of each emergence trap there will be a collection bottle filled with 150ml of a preservation solution consisting of a mixture of ethanol (70%), sucrose (40g/L), and glycerine (40ml/L) (Brock *et al.* 2010). All organisms will be counted and identified to the lowest practical taxonomic level.

Descriptive and multivariate methods of statistical analyses will be utilized (e.g., Biomass, Shannon's index, species abundances and Principle Response Curves) to assess both indirect and direct effects on the aquatic food-web. To illustrate the effects on important prey groups we will analyze the community based on functional groups such as infauna, benthos, herbivores/collectors, and predators.

Transport and fate of the pesticide mixtures: Samples of both water and sediment will be taken and analyzed to trace the presence of the pesticides and common breakdown products in each mesocosms tank. Sampling will be performed weekly for five weeks after pesticide application (34 samples per week, 170 samples in total for both water and sediment analysis). Two control samples will be taken before pesticide analysis. Water subsamples will be collected using amber pre-labeled, kilned and acid-washed glass bottles (950 mL). Sediment subsamples will be collected from the top 2 cm using pre-cleaned stainless steel spoons and carefully transferred to 950-ml amber pre-labeled, kilned and acid-washed glass bottles (Puckett 2002). Samples will be transported on wet ice to the laboratory, stored in the dark at 4°C and analyzed within two days of collection.

Sediment samples will be dried and ground. An aliquot of 10g of each sample will be used for further analysis. After adding 20 ml of a solution of hexane:ethyl acetate (1:1, v/v dried sediment will be sonicated for 30 min and centrifuged for 5 min. The solvent layer will be transferred into another centrifuge tube, and the sediment extraction will be repeated two times. All three solvent layers will be collected and combined. The combined extracts will be concentrated to 0.4 ml under a gentle stream of nitrogen and then extracted at a slow drip under vacuum using a GCB/PSA cartridge (Supelclean™ ENVI-Carb™ II), which was preconditioned using 3.0 ml hexane. The GCB/PSA cartridge will be used to effectively remove plant pigments such as chlorophyll, and plant sterols from the final extracts without the loss of planar compounds.

Water samples will be extracted using conditioned 6-ml solid phase-extraction C_{18} cartridge (Supelclean™ 500 mg, Sigma-Aldrich) at a slow drip under vacuum.
For sediment and water samples cartridges will be vacuum-dried for 2 hours to remove any excess water and frozen before further use. To elute pesticides columns will be rinsed twice with a 5-ml volume of a solution of hexane:ethyl acetate (1:1, v/v). Solvent elution (10 ml) will be collected and concentrated to 0.4 ml under a gentle stream of nitrogen. The internal standard

Dibromooctafluorobiphenyl (Chem Service, West Chester, PA) will be added to the concentrated extracts prior to GC analysis.

All final extracts will be analyzed using gas chromatography negative chemical ionization mass spectrometry (GC-NCI-MS) on Agilent 5973 series gas chromatograph (Agilent Technologies, Palo Alto, CA), equipped with a split-splitless injector (280°C, splitless, 1.5-minute purge time). The column will be a Supelco DB-5MS column (30 m x 0.25 mm with a 0.3 μm film thickness). Instrumental calibration will be performed using nine sets of calibration standard solutions with each pesticide (Chem Service, West Chester, PA), the surrogate Trans-Permethrin D6 (EQ Laboratories, Atlanta, GA), and the internal standard Dibromooctafluorobiphenyl in hexane. Quantification will be based on peak area using the standards.

5. Interaction with existing monitoring surveys or other studies:

Pesticide concentrations used in this study are based on preliminary tests carried out within the scope of the project "Effect assessment of tertiary pesticide mixtures on the amphipod *Hyalella azteca* and the midge *Chironomus tentans*" granted by the California State Water Resources Control Board to Dr. Connon and Simone Hasenbein (Agreement No. 06-447-300). This proposal is thus a natural succession from single species assessments to multiple-species interaction studies within mesocosms.

6. Feasibility:

The feasibility of this project is very high as all researchers involved are highly qualified and have the necessary experience to conduct the proposed study within the allocated time-frame. Dr. Connon has worked on contaminant-related issues over 10 years that have resulted in over 20 publications relating to ecotoxicology. Dr. Lawler has more than 20 years of experience in field studies and aquatic community ecology, and has over 50 peer-reviewed publications. The proposed methodologies are of common use in her laboratory and all the equipment needed to conduct this project is readily available. Simone Hasenbein is a PhD student that is fully funded (stipend and student fees) by the Bavarian Research Foundation, Germany. Mesocosm studies formed the core of her Master's thesis. She has taught undergraduate students in preparing, conducting and analyzing mesocosm studies, and has extensive knowledge on community toxicology and invertebrate taxonomy. Chemical analyses will be conducted in Dr. Thomas Young's laboratory (Department of Civil Engineering at UC Davis) where the proposed methods are of common practice. Simone Hasenbein has been trained by Dr. Young's staff to conduct the analyses of both water and sediment samples, and has conducted these assessments during our previous study "Effect assessment of tertiary pesticide mixtures on the amphipod *Hyalella azteca* and the midge *Chironomus tentans*" granted by the California State Water Resources Control Board to Dr. Connon and Simone Hasenbein (Agreement No. 06-447-300). Laboratory resources and equipment will be provided at no cost to this project.

7. **Deliverables:**

- We will provide quarterly progress reports which will describe the activities undertaken, accomplishment of the milestones, and any problems encountered in the performance of the work.
- A final report will be prepared to include a description of methods, all raw data and associated statistical analyses, results of all work to ensure QA/QC, and a discussion of the results including conclusions of the study. The final report will be submitted to the IEP 30 days after project completion (expected to be December 31st, 2013).
- This research will result in at least one peer-reviewed publication in a journal of significant impact; e.g. Environmental Science and Technology or other scientific journals with similar impact factors. Findings will be presented at scientific conferences. Targeted annual conferences include Delta Science Program related conferences and workshops, and Society of Environmental Toxicology and Chemistry (SETAC).

8. **Detailed budget and budget justification.**

Salaries		**Effort**	**Total wages**
R. E. Connon, Asst III Research Professor		5%	$3,555.00
Junior Specialist II		50%	$18,490.00
			$22,045.00
Benefits	**Total wages**	**Percentage benefit**	**Total benefits**
R. E. Connon, Asst III Research Professor	$3,555.00	30.3 % (2012-13) 33.3 % (2013-14)	$1,130.49
Junior Specialist II	$18,490.00	30.3 % (2012-13) 33.3 % (2013-14)	$5,879.82
			$7,010.31
Total Personnel			**$29,055.31**
Operating Costs			
Supplies			$13,500.00
Travel			$600.00
			$14,100.00
	Total w/o indirect costs		
Indirect Costs (Federal)	**$43,155.31**	54%	**$23,303.87**
		Federal total	**$66,459.18**

Overall Personnel: Included in the funds requested are 5% salary and benefits (total $4,685.49) for Dr. Richard Connon (PI) for project management activities including supervision of the project and training/assistance with data handling, report preparation and project oversight and outreach. Dr. Lawler (Department of Entomology, UC Davis) will act as a Co-PI at no cost to the

project, and will be involved as advisor and student co-supervisor, as well as in taxonomy and community classification, and the preparation of reports and publications. She will also provide equipment and some laboratory supplies in her lab at no extra cost.
We request fund to towards 50% salary and benefits for a Junior Specialist (total $24,369.82). The Junior Specialist will provide assistance with installing mesocosm tanks, invertebrate sampling and identification, as conducting chemical analyses.
Simone Hasenbein, the PhD student on this project, is fully funded for the entire study period, thus funds towards stipends and student fees are not requested.
Benefits rates are calculated based on current benefits cost as percentage of salaries.

Supplies: We request funds towards operating expenses amounting $13,500.00
Funding is required towards:

- Organic topsoil (1380 gallons in total)
- Artificial sediment, emergence traps and photo dishes for macroinvertebrate sampling
- Stainless steel tubes for zooplankton sampling
- Staining chemicals for zooplankton identification
- Specific extraction supplies such as solvents, chemicals and columns
- Pesticide formulations
- Hazardous waste disposal
- General supplies for laboratory use such as gloves, pipette tips and glassware,

Travel: Funds are requested to support domestic travel ($600.00 over one year) for team members to attend scientific conferences to present findings of the research. Targeted annual conferences include Delta Science Program related conferences and workshops, and Society of Environmental Toxicology and Chemistry (SETAC).

Overhead Costs: Federal funds carry a 54% overhead ($23,303.87).

Appendix

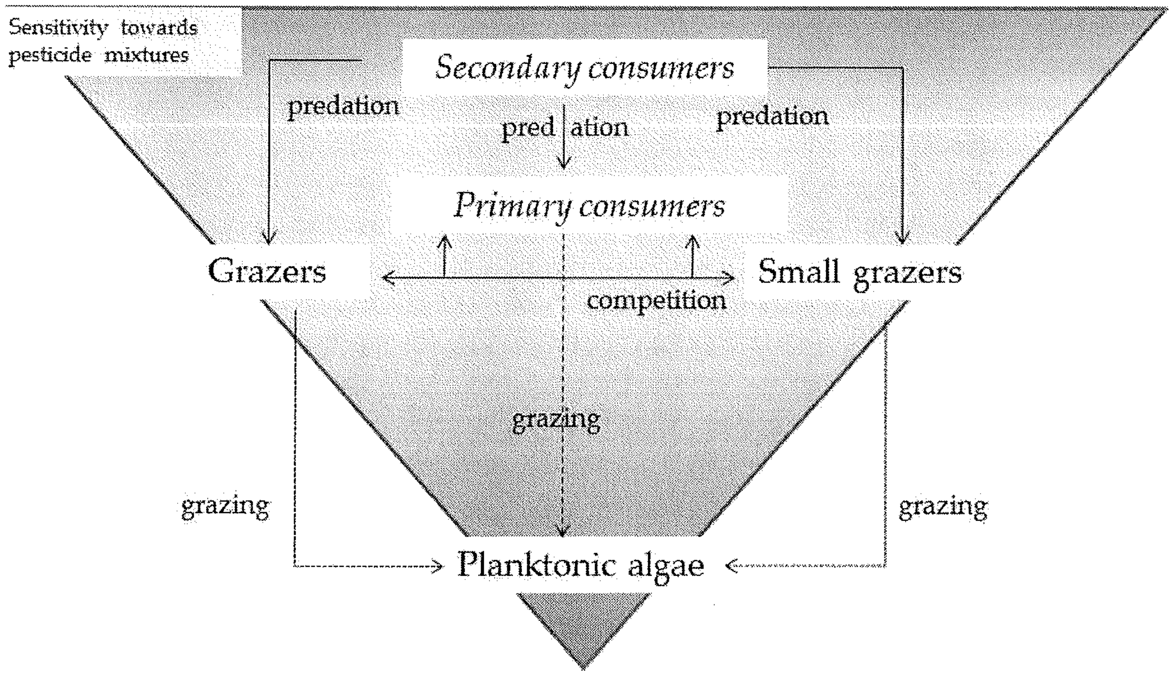

Figure 1 Conceptual model of the food web interaction under pesticide mixture influence (Hasenbein *et al.* 2010). The aquatic ecosystem incorporates five food web components to describe the trophic transfer of applied pesticide mixtures in an aquatic food web. These include, in increasing order of trophic level within the food web: planktonic algae, small grazers, grazers, primary consumers and secondary consumers. These components are not intended to represent discrete trophic levels, but rather generic levels of an aquatic food web (*e.g.*, primary producers, primary consumers, secondary consumers, and predators).

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