INVITED PAPER

PREDICTING 'NON-SCENTS': RATIONALE FOR A CHEMOSENSORY-BASED BIOTIC LIGAND MODEL

Greg Pyle[1]* and Chris Wood[2]

[1]Dept. of Biology, Nipissing University, North Bay, ON, Canada P1B 8L7.

[2]Dept. of Biology, McMaster University, Hamilton, ON, Canada L8S 4K1.

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COMMENTARY

Chemical communication systems involving pheromones, kairomones, and other signalling molecules, show a high degree of phylogenetic conservation in the underlying neurological organization (Metazoans) and molecular processing machinery among disparate extant species. These chemical communication systems are best developed and show the greatest diversity and sophistication in the aquatic environment, where organisms evolved under the selective influences of water—a universal solvent. Information conveyed to receiver organisms (especially animals) includes the location of predators (Chivers and Smith 1998; Wisenden 2000) or food (Valentinčič *et al.* 2000a; Valentinčič *et al.* 2000b), the identity of kin (Olsen *et al.* 1998; Courtenay *et al.* 2001; Mann *et al.* 2003), the genetic or reproductive status of potential mates (Eggert *et al.* 1999; Pinillos *et al.* 2002; Belanger *et al.* 2004), and the position of migratory routes (Hara 1994). In other words, chemical signals provide aquatic organisms with information related to the vital processes of life itself: finding food, avoiding becoming food, and reproduction.

Metals have long been known as powerful chemosensory inhibitors which thereby perturb natural behavioural patterns (Gardner and LaRoche 1973; Hansen *et al.* 1999a; Hansen *et al.* 1999b; Baldwin *et al.* 2003; Sandahl *et al.* 2004; Scott and Sloman 2004; Sandahl *et al.* 2006). Our recent work has shown that metals, at environmentally-relevant concentrations, can interfere with chemosensation in aquatic animals, both under controlled and natural conditions. Given the importance of chemosensation for maintaining healthy populations, metal-induced chemosensory deficits could potentially lead to large-scale ecological perturbations in metal-contaminated environments. Unfortunately, this effect has to date received only limited attention by the scientific community, and negligible attention by environmental regulators.

Using a range of aquatic animals from three trophic levels of a typical freshwater ecosystem, including leeches (*Nephelopsis obscura*) representing benthos, *Daphnia pulex* representing zooplankton, and fishes (including fathead minnows, *Pimephales promelas*, and yellow perch, *Perca flavescens*) representing predators, we have demonstrated subtle metal effects on chemosensation in every chemical communication system examined to date. Copper concentrations as low as 10 µg/L were sufficient to impair leech feeding activity (Pyle and Mirza 2007). Copper-exposed leeches were not attracted to a chemosensory food stimulus in a Y-maze, and spent more time searching for food and less time feeding than controls when presented with a food item in a feeding experiment. Adult *Daphnia pulex* exposed to 10 µg Cu/L were unable to produce neonates with neck spines (an inducible antipredator morphological defence mechanism) in the presence of a predator kairomone (Hunter and Pyle 2004), which significantly increased their vulnerability to predation and reduced their population growth rates relative to controls (Mirza and Pyle submitted, a, b). Fishes exposed to low aqueous metal concentrations could not respond to chemical alarm cue in the laboratory (Scott *et al.* 2003) or in the field (in metal-contaminated lakes around Sudbury) (McPherson *et al.* 2004), and were unable to differentiate between high- and low-condition mates on the basis of chemical cues in mate-choice experiments (compensation by visual cues did not occur; Hillman *et al.*, unpublished data). Although adult fish can usually recover from metal-induced chemosensory impairment after a short recovery period in clean water (Saucier and Astic 1995; Bettini *et al.* 2006; Sandahl *et al.* 2006), fish embryos exposed to environmentally-relevant metal concentrations suffer long-term or permanent chemosensory dysfunction (Carreau and Pyle 2005).

The biotic ligand model (BLM) has been developed as a regulatory tool to predict metal toxicity to aquatic organisms by considering site-specific water quality and metal binding dynamics at physiologically-sensitive binding sites or 'biotic ligands', which have traditionally been ionoregulatory proteins (channels, transporters, or enzymes) on the gills of fishes (Paquin *et al.* 2002; Niyogi and Wood 2004). These sites normally transport nutrient cations (e.g., Na^+, Ca^{2+}), and metal interaction with these sites manifests as "binding" in short term exposures (e.g., 3 hours). A considerable international research effort over the past decade or so has established a significant inverse relationship between the binding affinity (log K) of different metals to the gill surface and their acute toxicity – i.e., the aqueous metal exposure concentration required to kill 50% (LC50) of the test animals (Niyogi and Wood 2004). For each metal, and for several different organisms, specific log K values (negative log of the dissociation constant K for the gill ligand-ionic metal binding, at the 50% binding or toxicity point) have been determined experimentally, as well as binding site densities (B_{max}) and LA50 values (short term gill metal burdens predictive of 50%

*Author for correspondence, email: gregp@nipissingu.ca

mortality at 48-96 h). The protective actions of competing cations in the exposure water (e.g., Ca^{2+}, Mg^{2+}, H^{+}, Na^{+}) have been quantified as comparable log *K* values by their ability to inhibit gill metal-binding and (or) to raise the LC50. The protective actions of anions which may complex metals in the exposure water [e.g., Cl^{-}, HCO_3^{-}/CO_3^{2-}, natural organic matter (NOM) or dissolved organic carbon (DOC)] have been similarly quantified. With the help of geochemical modelling programs, Biotic Ligand Models have been built using these constants to predict gill metal burdens, and therefore toxicity for diagnostic organisms, as a function of the known chemistry of specific receiving waters. The BLM has shown great promise as a regulatory tool given its conceptual simplicity and that its predictions correspond with measured toxicity within a factor of approximately plus or minus two-fold in extensive acute toxicity testing with natural waters (Santore *et al.* 2002). Consequently, the BLM approach has been adopted or considered for adoption by several countries/jurisdictions around the world, including Australia, Canada, the European Union, New Zealand and the United States.

However, predictions from the traditional gill-based BLM approach involve several assumptions which have been difficult to reconcile under natural conditions. The model assumes that water is the only source of metal available to the organism, despite increasing evidence that dietary metals contribute significantly towards fish tissue metal accumulation (Clearwater *et al.* 2002; Meyer *et al.* 2005), including at the gills (Mount *et al.* 1994; Kamunde *et al.* 2002). Dietary metals can accumulate to a higher total concentration in the gill before toxicity is induced relative to waterborne metals, probably because waterborne metals are taken up via apical ion channels and transporters in their most bioavailable and toxic form (i.e., Me^{n+}), whereas dietary metals are probably protein-bound and relatively non-toxic by the time they enter the gill tissue through basolateral pathways (Kamunde *et al.* 2001). Because a gill-based BLM (gbBLM) attempts to predict toxicity based on the concentration of metals in the gill, the relative contribution of waterborne and dietary metals to total gill metal concentration and toxicity must be known before accurate predictions can be made.

Another issue is that B_{max}, log *K*, and LA50 values can be affected by dietary ion and metal content, acclimation water quality conditions, and exposure history (Niyogi and Wood 2003; Franklin *et al.* 2005). Although one assumption of a gbBLM is that metal-gill binding constants are fixed for any given organism, several studies have demonstrated that this is not the case—especially in wild fish subjected to chronic exposure in metal-contaminated lakes (Taylor *et al.* 2000; Niyogi *et al.* 2004; Klinck *et al.* 2007; Niyogi *et al.* 2007; Pyle and Wood in press). These observations have made it difficult for the BLM to predict metal toxicity to wild fish inhabiting metal contaminated freshwater systems.

At present, gbBLMs are developed to predict acute toxicity. However, most natural metal-contaminated systems rarely achieve metal concentrations that are high enough to cause acute toxicity. In recent surveys of several lakes around the metal-mining district of Sudbury, Ontario, Canada, measured aqueous metal concentrations were considerably below concentrations required to induce acute toxicity to resident fish (Pyle *et al.* 2005; Couture *et al.* 2008), which calls into question the ecological relevance of gbBLM predictions of acute toxicity. To address some of these shortcomings of the current BLM paradigm, research emphasis has begun to shift from predicting acute toxicity to chronic toxicity, in part by acknowledging the influence of metal-gut dynamics (Klinck *et al.* 2007; Niyogi *et al.* 2007), the interaction between waterborne and dietary metals (Kamunde *et al.* 2002), and the role of diet quality on metal uptake and accumulation in gills (Zohouri *et al.* 2001; Kamunde *et al.* 2003; Pyle *et al.* 2003; Baldisserotto *et al.* 2004; Kjoss *et al.* 2005).

The cornerstone of any model that attempts to predict toxicity is ecological relevance. Because of some of the issues raised above, especially the influence of diet on gill-metal accumulation and the focus on acute toxicity, the current gbBLM has been difficult to validate in wild fish inhabiting metal-contaminated environments. One approach to overcoming these issues is to develop a chemosensory-based BLM (cbBLM) for laboratory-reared and wild fishes, using the olfactory epithelium as the biotic ligand. Like the gill epithelium, the olfactory epithelium is continually exposed to ambient water throughout a fish's life. Unlike the gill epithelium, the primary function of olfactory epithelium is to truly *bind chemicals* (rather than transport them) in the environment (usually odour molecules), making it ideally suited for biotic ligand modelling purposes. Waterborne metals certainly accumulate in the olfactory epithelium (Julliard *et al.* 1995; Scott *et al.* 2003). Binding constants generated from metals binding to the olfactory epithelium should not be affected by dietary metals because dietary metals are unlikely to accumulate in the olfactory epithelium (Scott *et al.* 2003).

We have now developed a method using radiolabelled metals for calculating BLM parameters log *K* and B_{max} at the olfactory epithelium (Mirza *et al.* submitted). Our early results indicate that metal-binding to the olfactory epithelium is saturable, at least for Cd, suggesting that a discrete number of sites are involved. Membrane saturability by metals is a requirement for the successful calculation of BLM parameters. Moreover, wild fish from metal contaminated lakes show an increase in metal-olfactory epithelium binding capacity (B_{max}) and a decrease in binding affinity (log *K*), which is directly analogous to observations in wild fish gills. We are currently in the process of relating metal effects, such as neurophysiological responses (measured using electro-olfactography (EOG); e.g., Sandahl *et al.* 2004; Sandahl *et al.* 2006) of the olfactory epithelium to standard chemosensory stimuli and behavioural responses to the same stimuli, to BLM binding parameters. Progress to date suggests that development of a cbBLM using the fish olfactory epithelium is possible (e.g., Sandahl *et al.* 2007).

Implementing a cbBLM will pose some logistical challenges. Chemosensory epithelia are typically small relative to gills and the metal concentrations required to induce a chemosensory effect are low. These small samples sizes and low metal concentrations have necessitated the use of radioisotopes in order to estimate BLM parameters log K and B_{max}. Although we have begun developing neurophysiological methods analogous to EOG for daphniids (i.e., electro-antennogram; EAG), the same analytical issues related to small sample size will need to be overcome. New, non-radiological methods should be developed that can accommodate small tissue biomass and low metal concentrations.

The potential advantages of a cbBLM over a gill-based BLM are many-fold. Ecologically-important metal effects related to chemosensation, such as finding food and mates and avoiding predators, are known to occur at environmentally-relevant, sub-acute metal concentrations (Pyle and Mirza 2007). Results generated in the laboratory can be extrapolated to the field (McPherson *et al.* 2004). Chemosensory epithelia serve only one function—binding molecules. Consequently, confounding factors related to the source of metal exposure (aqueous vs. diet) are likely mitigated in a chemosensory-based model. Moreover, because of the high degree of phylogenetic conservation of the molecular machinery that drives chemosensation among disparate animal groups (Hildebrand 1995), identification of a single mechanism of toxic action may be extrapolated to a wide range of species. Ultimately, a cbBLM may prove to be an important contribution towards improving the ecological relevance of Environmental Risk Assessments in metal-contaminated environments. Our next challenge will be to link cbBLM predictions to large-scale population, community, or ecosystem effects.

SUMMARY AND CONCLUSIONS

An increasing body of research has provided unequivocal evidence that dissolved metals can interfere with chemosensation at or below concentrations typically associated with other toxicity endpoints. These effects have the potential to result in large-scale ecological perturbations. The Biotic Ligand Model (BLM) attempts to predict acute metal toxicity by considering site-specific water quality and metal binding characteristics at a physiologically-sensitive biotic ligand, which is typically the gills of fishes. However, several factors are now known to influence gill metal-binding dynamics, including metal concentrations and ionic composition of the diet. Moreover, gill-based BLMs generally predict acute metal toxicity, but metals in contaminated natural waters rarely achieve concentrations high enough to cause acute toxicity. These confounding factors have made it difficult to apply a gill-based BLM approach to wild fishes inhabiting metal-contaminated freshwater environments and have led to questions about ecological relevance of BLM predictions. Here, we propose to substitute the olfactory epithelium for the gill epithelium as a biotic ligand for BLM purposes in support of a chemosensory-based BLM (cbBLM). Although there may be some analytical challenges to overcome owing to small tissue biomasses associated with chemosensory structures, the advantages of such a BLM approach could improve the ecological relevance of its predictions over the current gill-based paradigm.

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