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Protein model for pollutant uptake and elimination by living organisms and its implications for ecotoxicology

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ABSTRACT: The conceptual model on which chemical assessment of pollutants is based is flawed. The assumption in ecotoxicology—that pollutants cross the biological membrane only by passive diffusion of their solute phase (diffusion model) and thus, that only water-soluble pollutants are biologically available—is inconsistent with the biologists' understanding of the role and functioning of the biological membrane. The biological membrane both isolates organisms, cells and organelles from their external environments and regulates cross-membrane trafficking of polar and non-polar substances. Trafficking regulation is a function of proteins dissolved within the membrane. An alternative protein model for pollutant uptake and elimination is proposed that provides a credible explanation of how solid-phase pollutants, such as those bound to aquatic sediments and soils, may be readily incorporated by living organisms. Current chemical testing is likely to underestimate the risk posed to organisms by sediment and soil-bound pollutants. New techniques for assessing the bioavailability and impacts of pollutants, based on the protein model, are urgently needed.

KEY WORDS: Ecotoxicology · Pollution · Protein model · Diffusion model · 3-phase model · Cell membrane · Equilibrium partitioning coefficient · Transport proteins · Sediment · Pore water

INTRODUCTION

Our interest in ecotoxicology was sparked when standard chemical tests of sediments failed to explain a widespread and sustained collapse of macrobenthic infauna (animals ≥0.5 mm living in aquatic sediments) in riverine estuaries of SE Queensland, Australia. Their catchments have become highly urbanised over the last 30 yr. Studies from 1972 to 1974 found that these estuaries supported macrobenthic infaunal densities of up to 35 000 m^{-2}, with mean abundances of ~4000 m^{-2} (Campbell et al. 1974a,b,c, Stephenson & Campbell 1977). More than 80% were deposit-feeders. In the same estuaries today, infaunal abundances are <2.5% of their former values. Suspension-feeders have been less impacted than deposit-feeding organisms, but are also absent or rare in those estuaries with the largest urban/industrial catchments. Estuaries with lower vulnerability to catchment pollution have impoverished sediment infauna, but continue to support an abundance of macrobenthos on logs that protrude above the sediments (S. Quinnell unpubl. data). These observations led us to postulate that the faunal loss was the result of contaminated sediments, rather than of water pollution. When extensive chemical testing of the sediments, based on internationally accepted protocols, failed to identify any causal agent (Miller 2000), we were forced to question whether sediment contamination was being appropriately assessed, especially the threat posed by pollutants bound to sediments and organic matter ingested by deposit-feeding infaunal organisms.

Sediments in aquatic ecosystems accumulate pollutants, with concentrations often many orders of magnitude greater than in associated water (Meador et al. 1995, Nipper 2000, Anderson et al. 2001, Burd 2002, Fent 2003). In aquatic ecosystems, benthic organisms are well-suited indicators of environmental contamina-

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tion (University North Carolina 1994, Krantzberg et al. 2000, Anderson et al. 2001), because their usually restricted mobility makes it difficult for them to escape contaminated conditions (Peso-Aguiar et al. 2000). Sediment-sorbed contaminants may profoundly impact biological communities (Morissey et al. 1996, Ferraro & Cole 1997, Grumiaux et al. 1998, Burd et al. 2000, Peso-Aguiar et al. 2000, Stewart et al. 2000, Xu et al. 2002), with adverse ecological changes accumulating through time (Grumiaux et al. 1998). Such changes may be indicators of adverse toxic effects not identifiable by chemical tests (Stewart et al. 2000, Roach et al. 2001).

Chemical testing alone is known to be inadequate for environmental quality assessment. Some pesticides presently in use, including the synthetic pyrethroids, are lethal to fishes at sediment pore (interstitial) water concentrations <0.2 ppb (Siegfried 1993) and to invertebrates (especially arthropods) at <1 ppb. This is below the standard minimum detection threshold of pore water concentrations of most organic pollutants (≥1 ppb) (Neal et al. 1998, Stewart et al. 2000, Roach et al. 2001). Sublethal levels of toxicity that may impact food webs (Drenner 1993) are likely to be much lower (Siegfried 1993). Scientific frameworks for evaluating the ecological significance of contaminants in sediments are 'either lacking or not widely used or communicated' (Krantzberg et al. 2000, p. 388).

Ecotoxicological modelling of pollutant behaviour in ecosystems is based on the hypothesis that pollutants enter and leave living organisms (i.e. cross the external biological membrane) only by passive diffusion of their solute phase through the membrane's phospholipid bilayer (the 'diffusion model'). The corollary is that only pollutant solutes are available for uptake by living organisms (e.g. Chiou et al. 1982, Hawker & Connell 1985, 1986, 1988a,b, Connell 1988, 1990, Markwell et al. 1989, Di Toro et al. 1990, Just et al. 1990, Stenerson 1992, De la Torre et al. 1995, Karrupiah & Gupta 1996, Skoglund et al. 1996, Mitra & Dickhut 1999, Carbonell et al. 2000, Chu & Chan 2000, Maskaoui et al. 2002, Nipper et al. 2002, Williamson et al. 2002, Fent 2003). This assumption ignores an inherent primary function of the bilayer of the biological membrane—that it is an impermeable barrier which isolates any living unit (organelle, cell or living organism) from its external environment (e.g. Alberts et al. 1994, Voet et al. 1999, Madigan et al. 2003). Because of the bilayer's impermeability, living organisms can sustain a chemically oxidising internal environment of high complexity in a state of non-equilibrium with the chemically reducing, less complex external environment. The impermeability of the biological membrane to passively diffusing materials also allows cells to sustain and regulate high internal concentrations of thousands of types of often very complex organically synthesised molecules (Allen & Starr 1982, Alberts et al. 1994, Depwer & Weber 1996, Voet et al. 1999, Madigan et al. 2003, Williams & Fraústo Da Silva 2003). Of necessity, cross-membrane substance trafficking (movement) in both prokaryotes (Madigan et al. 2003) and eukaryotes (Alberts et al. 1994, Voet et al. 1999) is governed by proteins dissolved within the membrane. Thus, the diffusion model is inconsistent with contemporary biological understanding. It cannot accurately assess risks posed by pollutants to living organisms, especially those pollutants that are readily sorbed onto organic particles in sediments or suspended in water, and incorporated via ingestion. Prime candidates for such uptake are insecticides, many organic industrial chemicals, and many heavy metals.

The concept of protein-regulated cross-membrane trafficking of substances is well established in the biological sciences, including molecular biology, biochemistry and medicine, but has not previously been applied in ecotoxicology. We here propose that the uptake, internal transport and elimination of all pollutants by living organisms is regulated by proteins dissolved within biological membranes, and by intracellular transport proteins. This 'protein model' must be urgently incorporated into the conceptual framework for ecotoxicological research. While not based on original experimental data, our work does draw upon an extensive literature from biochemistry, molecular and cell biology, genetics, pharmacology, anaethesiology, oncology, physiology, ecology and marine biology, as well as ecotoxicology.

In this paper, we first consider ecotoxicology's diffusion model. Secondly, evidence from other models that might explain cross-membrane transport of molecules is evaluated. Thirdly, the protein-transport mechanism from cell biology is applied to the uptake and elimination of pollutants and their metabolites by living organisms. Finally, we present some of the implications of the protein model and its application for the conduct and validity of ecotoxicological studies, with emphasis on aquatic environments. This paper's primary theme is the fates of organic contaminants, with less emphasis on heavy metals to limit text length. The environmental fates of heavy metal contaminants will be protein-regulated in the same general manner as organic pollutants.

MODELS

Theory and application of 'diffusion model' in ecotoxicology

The 'diffusion model', first developed about 20 yr ago by chemists and engineers under the guise of parti-

tioning coefficients (K), is employed to explain movement and accumulation of pollutants between and within primary storage sites (e.g. air, aquatic sediments, soils, water and living organisms). Accordingly, partitioning is determined entirely abiotically by concentration gradients between storage sites of the contaminant's solutes (see Fig. 1) (e.g. Hawker & Connell 1985, 1986, 1988a, Connell 1988, Coats et al. 1989, Connell & Bycroft 1990, Just et al. 1990, De la Torre et al. 1995, Murphy et al. 1995, Karrupiah & Gupta 1996, Hamer et al. 1999, Mortimer et al. 1999, Chu & Chan 2000, Anderson et al. 2001, Hendriks et al. 2001, Nipper et al. 2002, Fent 2003). While the diffusion model is based on principles of physical chemistry that have been validated for abiotic environments, it is of limited value when applied to biota because it does not allow for the biological membrane's ability to isolate and regulate substance uptake, nor its capacity to actively export substances, including many toxins (see later subsection 'Why diffusion is not the process …'). Partitioning coefficients are favoured because they are simple, quick, and inexpensive to measure, and use generalised equations to extrapolate the risks posed by thousands of toxic substances to millions of species (Hendriks et al. 2001).

Under the diffusion model, the focus in ecotoxicological studies is on concentrations of pollutant solutes in the water column and substrate pore waters in aquatic and soils (e.g. Hawker & Connell 1985, 1986, 1988a,b, Connell et al. 1988a,b, Markwell et al. 1989, Connell 1990, Connell & Bycroft 1990, Di Toro et al. 1991, Murphy et al. 1995, Skoglund et al. 1996, Van Beelan & Fleuren-Kemila 1997, Hamer et al. 1999, Camusso et al. 2000, Collavini et al. 2000, Peso-Aguiar et al. 2000, Berglund et al. 2001, Nipper et al. 2002). For aquatic ecosystems, it has been stated that: 'With the exception of a limited number of highly lipophilic pollutants, waterborn exposure is considered the most relevant exposure route for aquatic organisms … some consider … only the dissolved fraction is bioavailable for pelagic organisms' (Carbonell et al. 2000, p. 107), and that '… pore water provides a direct measure of contaminant exposure to aquatic organisms because it incorporates the physical and chemical parameters that affect bioavailability' (Nipper 2000).

Thus, concentrations of sediment and soil-bound contaminants are not used as direct indicators of pollution threats, despite the knowledge that (1) >99.99% of highly lipophilic pollutants may be associated with sediments in aquatic ecosystems (e.g. Meador et al. 1995, Hamer et al. 1999, Chu & Chan 2000, Nipper 2000, Anderson et al. 2001, Burd 2002, Maskaoui 2002, Smith & Pritchard 2002, Fent 2003, Van der Oost et al. 2003), (2) sediments, therefore, are major repositories for organic pollutants (e.g. Tagatz et al. 1987, Meador et al. 1995, Hamer et al. 1999, Chu & Chan 2000, Nascimento et al. 2000, Nipper 2000, Peso-Aguiar et al. 2000, Anderson et al. et al. 2001, Wong et al. 2001, Burd 2002, Lahr et al. 2002, Maskaoui 2002, Smith & Pritchard 2002, Vijver et al. 2002, Fent 2003, Van der Oost et al. 2003), (3) sediments also may store high concentrations of heavy metals (e.g. Birch et al. 2000, Tilquin et al. 2000, Burton et al. 2001, Ellis et al. 2001, Burd 2002, Zabetoglou et al. 2002), and (4) that soils are major storage sites in terrestrial ecosystems (e.g. Chu & Chan 2000, Hund-Rinke & Kordel 2003).

Many sediment-bound organic pollutants, notably persistent organic pollutants (POPs) (e.g. Hawker & Connell 1986, Connell 1988, Zhou et al. 2000, Guruge & Tanabe 2001), and many heavy metals, are also highly stable in the environment (e.g. Lawrence & Mason 2001, Doyle et al. 2003, Peltier et al. 2003). Such longevity enhances a contaminant's bioconcentration and bioaccumulation in biota (e.g. Hawker & Connell 1986, Connell 1988, Weis & Weis 1992, Munoz et al. 1996, Peters et al. 1999, Ruus et al. 1999, Dang Duc Nhan et al. 2001, Fisk et al. 2001, Lawrence & Mason 2001, Senthilkumar et al. 2001, Scheifler et al. 2002a,b, Weltje et al. 2002, Hursthouse et al. 2003, Van der Oost et al. 2003). Currently, bioconcentration and passage of pollutants through food webs is interpreted as solute-phase diffusional uptake in all trophic-level interactions (e.g. Schramm et al. 1998, Peters et al. 1999, Ruus et al. 1999, Carbonell et al. 2000, Fisk et al. 2001, Weltje et al. 2002). Biomagnification along the food chain is thought to occur via transfer of solute pollutants from food within the intestinal lumen of animals (e.g. Fisk et al. 2001, Fent 2003, Van der Oost et al. 2003). This ignores the fate of pollutants bound to food macromolecules, which are actively transported across the membrane of gut-wall epithelial cells.

The 3-phase model

In tests for lipophilic pollutants in aquatic ecosystems, the diffusion model is represented by the '3-phase model' (e.g. Hawker & Connell 1985, 1986, 1988a,b, Baughman & Perenich 1988, Markwell et al. 1989, Just et al. 1990, De la Torre et al. 1995, Karrupiah & Gupta 1996, Hamer et al. 1999, Mitra & Dickhut 1999, Chu & Chan 2000, Berglund et al. 2001, Nipper et al. 2002, Skrabal & Terry 2002, Williamson et al. 2002) (Fig. 1). Protocols for its application specify 3 locations for pollutants—sediments, water and lipids (i.e. living organisms) (Hawker & Connell 1986, 1988b, Markwell et al. 1989, De la Torre et al. 1995, Mitra & Dickhut 1999, Nipper et al. 2002, Williamson et al. 2002). Although it was not originally intended for such, the 3-phase model has been extended to studies of pollutants in

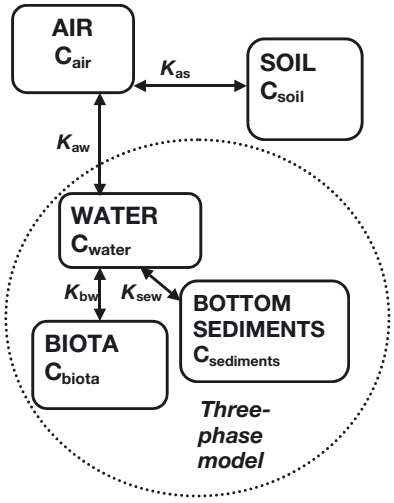

Fig. 1. 'Diffusion model' incorporating '3-phase model' for a toxic chemical in a river system. C: concentrations at different sites for air (a), soil (s), water (w), biota (b) and sediments (se); *K*: partitioning coefficient between sites (after Connell & Bycroft 1990, p. 313). Note there is no direct connection between contaminants in sediments and biota

terrestrial ecosystems (e.g. Stenerson 1992, Trapp et al. 2001, Hund-Rinke & Kordel 2003, Scheifler et al. 2003) and to include heavy metals (e.g. Collavini et al. 2000, Lahr et al. 2002, Datry et al. 2003, Scheifler et al. 2003) and other ionic pollutants (e.g. Zabetoglou et al. 2002). Under the 3-phase model, concentrations of lipophilic pollutants in tissues depend on abiotic partitioning coefficients (K_{ow}) between the solute (w) and lipid phase, which in laboratory tests is often represented by octanol (o) (e.g. Hawker & Connell 1988a,b, Coates et al. 1985, Chu & Chan 2000). Thus, global concern about 'water pollution' levels is a reflection of the 'solute is the active phase' concept of ecotoxicology's 3-phase model, and downplays the potentially great direct environmental impact of sediment-bound contaminants. Similarly, in terrestrial ecosystems, application of the 3-phase model to studies of pollutants has led to substantial concern about contamination of ground-water and, with less immediate concern, about the risks of soil-bound contaminants. Other partitioning coefficients also may be employed, notably a soil/sediment partitioning coefficient and a water-solubility coefficient (Periera et al. 1988). All are based on the principle of passive diffusion.

Abiotic partitioning coefficient (K_{ow}) tests

Application of the diffusion model includes the use of *ex vivo* tests to determine partitioning coefficients. In these, living organisms are replaced by a functional substitute (usually octanol), sometimes with an artificial semipermeable membrane device (SPMD) to partition water and the test lipid or lipid substitute (e.g. Di Toro et al. 1990, 1995, Carbonell et al. 2000, Williamson et al. 2002). The SPMD consists of a polymeric membrane that encloses a model lipid, and acts as a passive integrative sampler for determining the aqueous concentration of hydrophobic contaminants (Williamson et al. 2002).

Some ecotoxicological studies involving highly lipophilic pollutants have shown that K_{ow} approximates the measured water: living organism partitioning for highly lipophilic compounds (e.g. Hawker & Connell 1988a, Periera et al. 1988, Connell et al. 1993, Mitra & Dickhut 1999, Mirtagotri 2002). Such apparent affirmation of the 3-phase model has resulted in its uncritical acceptance. In contrast, many studies have also found that partitioning coefficients do not explain experimental outcomes, even for highly lipophilic materials (e.g. Maskaoui 2002, Zabetoglou et al. 2002) and that K_{ow} does not explain observed partitioning based on tissue loads and water concentrations (Moriarty 1988, Halling-Sørensen et al. 2000, Maskaoui, 2002, Fent 2003) and, predictably, that tissue loads are often more useful for assessing contamination than K_{ow} (Moriarty 1988). Agreement between tissue pollutant loads and a K_{ow} for some chemicals simply demonstrates that highly lipophilic chemicals are readily and preferentially deposited or sequestered in adipose tissues of the test organisms via transport proteins. This will be especially true if an organism lacks the transport proteins necessary for elimination of a particular lipophilic pollutant. Even more questionable, given the biochemical and physiological variability between living organisms, has been the practice of extrapolating the findings from studies of K_{ow} on one organism to other species (Hendriks et al. 2001).

Concern that the 3-phase model under-represents the complexity of aquatic pollutant chemistry led to application of the 'critical micelle concentration' concept for assessing environmental fates of lipophilic pollutants (Di Toro et al. 1990, Park et al. 2002). This concept is highly applicable to understanding encapsulation, concentration and transport of pollutants in aqueous environments (see later subsection 'Delivery of pollutants to receptor proteins'), but was not extended to the delivery and uptake of pollutants across biological membranes.

Why diffusion is not the process — function of biological membrane

For a living biological organism to consist of cells enclosed by semipermeable membranes is contrary to

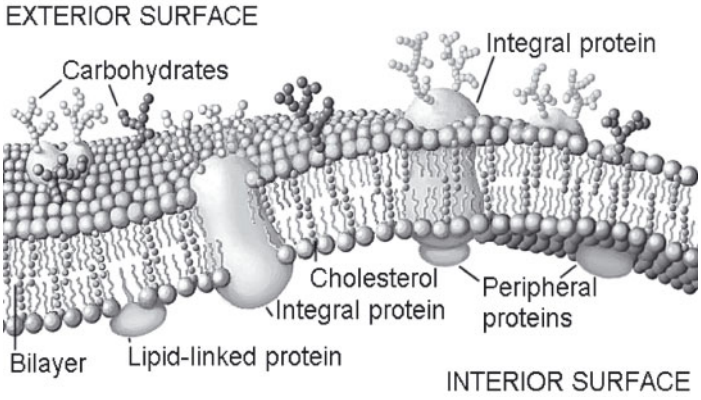

Fig. 2. Biological membrane showing proteins embedded in phospholipid bilayer. Integral proteins includes transport proteins. Lipid-linked proteins mediate protein–protein interactions or modify the functions of other proteins with which they associate. (Illustration Sally Elmer adapted from Alberts et al. 1994 and Voet et al. 1999)

its intrinsic need to maintain specific internal homeostasis (Depwer & Weber 1996). The biological membrane is an amphiphilic (i.e. simultaneously hydrophilic and hydrophobic) asymmetrical bilayer of phospholipids, about ~60 Å thick, containing ~5×10^6 lipid molecules μm^{-2} (Alberts et al. 1994, Voet et al. 1999). Within the bilayer are regulatory proteins and cholesterol molecules (membranes may have as many cholesterol units as phospholipids) that enhance the bilayer's impermeability (Alberts et al. 1994) (Fig. 2). The membrane's outer surface bristles with carbohydrates, whose functions are still being unravelled. In eukaryotes the membrane is assembled in the endoplasmic reticulum (ER), where basic membrane asymmetry is generated according to each cell's needs (Alberts et al. 1994, Voet et al. 1999, Camus et al. 2000).

The 'formidable' impermeability (Alberts et al. 1994) of the biological membrane is conferred by the structure of the membrane's bilayer. Because they are charged (hydrophilic), phospholipid 'heads' interact readily with water, forming external and internal membrane surfaces that are highly lipophobic and, therefore, powerful barriers to diffusing solutes of non-polar (lipophilic) substances. In addition, diffusion of non-polar materials towards the membrane is inhibited by a zone of still water up to 2 nm thick, covering both membrane surfaces (Alberts et al. 1994). Phospholipid 'tails', in contrast, are non-polar and form the double inner lipophilic zone that is a powerful hydrophobic barrier to diffusing solutes. The impermeability of the biological membrane is such that even protons (H^+) cannot diffuse across it (Madigan et al. 2003). Substances cross the living biological membrane only through the activity of proteins embedded within the membrane.

Alternative mechanisms for cross-membrane transport

Given the impermeability of the biological membrane, only 2 mechanisms currently known in molecular biology can be invoked to explain cross-membrane trafficking of pollutants by living organisms, i.e. 'flip-flops' (for complex molecules that may embed in the lipid bilayer of the membrane) and protein transport.

The flip-flop has been proposed as the mechanism by which phospholipids transfer from one side of the biological membrane to the other (Alberts et al. 1994). A study of oleic acid (an essential lipid-soluble fatty acid) indicated that oleic acid binds with the lipid domain of the membrane and is transferred across the membrane within 5 s via a flip-flop (Kamp et al. 2002). Because the study also showed that oleic acid had a partitioning coefficient similar to that in synthetic phospholipid bilayers, the flip-flop has been considered as a possible explanation of pollutant trafficking across the membrane. In synthetic lipid bilayers, spontaneous flip-flop occurrence is <1 mo^{-1} $molecule^{-1}$, leading to the general consensus that spontaneous flip-flops are extremely rare (Alberts et al. 1994, Voet et al. 1999). Instead, enzymatic proteins (flipases) catalyse the transfer of essential materials, including fatty acids, across the membrane. Flipases are highly substrate-specific and it is most unlikely that any would transfer pollutants. They are also active mainly within the cell's ER, which should not be readily exposed to toxins originating outside the cell. Therefore, flip-flops, which may allow very slow rates of abiotic uptake of molecular units that successfully embed in the bilayer, cannot explain the high rates of cross-membrane trafficking of pollutants (e.g. 'knock-down' insecticides).

'Protein model' for pollutant transport

A 'protein model', whereby cross-membrane trafficking of substances is mediated by an array of specific proteins embedded in the biological membrane, appears to be the only viable explanation available now for uptake and elimination of pollutants by living organelles, cells, tissues and organisms. Identification of the regulatory proteins is revolutionising cell-membrane biology. Quite recent text books (e.g. Voet et al. 1999) state that water diffuses across the cell membrane; however, aquaporins and an increasing number of other proteins have been identified as regulators of cross-membrane water transport (Chaumont et al. 2001, Garcia et al. 2001, Sansom & Law 2001, Zeuthen

et al. 2001, Konzo et al. 2003, Madigan et al. 2003). Protein transporters also have been found for other small molecular units, including NH_3 (Hu & Wu 2001) and NO (Closs et al. 2000, Stevens et al. 2000). Internal transport proteins also are involved in internal movements of substances, including lipidic molecules (e.g. O_2 by haeme-proteins) (Van De Graaff & Fox 1992, Alberts et al. 1994), whose insolubility in water results in limited amounts being available via passive diffusion.

Cell protein diversity

About 10^9 proteins of ~10^4 types occur in a typical mammal cell (Alberts et al. 1994). Other eukaryotic cells probably have comparable numbers and kinds of proteins. Some proteins are structural, many are functional (e.g. catalysts and transporters). Designation of locations and concentrations of transport proteins in eukaryotes (Alberts et al. 1994) is a factor in specialisation of organelles, cells, tissues, organs, individuals, and even species. In mammalian nerve-cell myelin sheaths, whose primary function is electrical insulation, less than 25% of the membrane mass is protein, whereas proteins form about 75% of membrane biomass in membranes whose function is energy transduction (e.g. mitochondria) (Alberts et al. 1994). Proteins may collaborate in trafficking regulation. Pores in the membrane of eukaryotic cell nuclei, which govern movement of molecules into and out of the nucleus, contain up to 1000 proteins of 50 to 100 kinds (Voet et al. 1999). Proteins are synthesised in the cell's ER and, like membrane lipids, once in place membrane proteins do not flip-flop across the bilayer (Alberts et al. 1994).

Transport proteins have been grouped into a number of families based on fundamental chemical structure. A few of these protein families seem to have arisen recently in evolutionary time (Ahearn et al. 2001, Paden et al. 2001), but genome analyses show that most proteins appear to belong to a limited number of archaic family groupings that have diversified into an array of kinds (Quintero & Blatt 1997, Paden et al. 2001, Petit et al. 2001, Konzo et al. 2003). The YCF 1 transport protein, which eliminates Cd^{2+} from yeasts, is very similar to a protein that eliminates Cd^{2+} from insect cells (Ren et al. 2000). Therefore, studies of protein functions in one organism type (e.g. yeasts or fishes) may be useful in understanding similar functions in other types of organisms (e.g. insects, mammals and flowering plants). The development of transport proteins must have been as critical in the evolution of the primordial cell ~3.5 billion yr ago as formation of the biological membrane and the assembly of nucleic acids.

How transport proteins mediate cross-membrane trafficking

Transport proteins function in 2 basic ways, as channels or carriers. Channel proteins form temporary aqueous pores that open and close according to a cell's requirements. Carrier proteins bind to molecular units for transport without chemically transforming them (Madigan et al. 2003), then pass across the lipid bilayer or structurally reconfigure to release the transported substance on the other side of the membrane (Voet et al. 1999). All transport proteins identified so far have been highly substrate-specific. Valinomycin, a bacterial channel protein, accommodates K^+ (r = 1.33 Å), but not Na^+ (r = 0.95 Å) or Li^+ (r = 0.60 Å) (Alberts et al. 1994). In *Escherichia coli*, the OmpF porin (channel protein that carries low molecular weight, ionic substances) forms a weakly positively charged channel, that admits only anions <600 Å, whereas the structurally similar PhoE has a weakly negative changed channel that carries only small cations (Voet et al. 1999). Protein expression in membranes can be determined by environmental conditions. In *E. coli*, the osmolarity of the environment determines whether the OmpF or the OmpC is synthesised in the bacterium's outer membrane (Madigan et al. 2003).

Channel proteins

Channel proteins form aqueous pores extending across the membrane bilayer. These function in a 'gated' (open–closed) manner (Alberts et al. 1994) (Fig. 3). When open, channel proteins allow 'facilitated diffusion' of materials down concentration or electro-

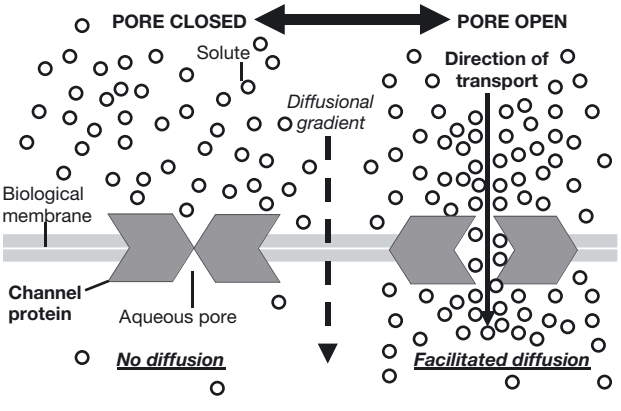

Fig. 3. Diagrammatic representation of channel protein functioning. Protein operates in a gated (open–closed) manner according to a cell's or organelle's needs. Open proteins allow solutes to move along their concentration or electrochemical gradients; such proteins transport only small ions. (Illustration S. Quinnell adapted from Alberts et al. 1994 and Voet et al. 1999)

chemical gradients according to a cell's needs, closing once those needs are met. Evidence suggests that only small ions are transported in this manner (e.g. H_2O, Cl^-, K^+, Na^+) (Alberts et al. 1994) and that channel proteins are highly selective of substances for transport. Solutes as small as Fe^{2+} and Fe^{3+} may employ carrier proteins. Therefore, channel proteins may not be involved in cross-membrane trafficking of pollutants except, perhaps, of some very small ionic units (e.g. NH_4^+).

Subtle changes to protein trafficking can have profound metabolic effects (Stewart at al. 2000). The antibacterial drug Gramacidin A is a channel protein of 15 amino acid residues. When inserted into a bacterium's membrane, Gramacidin A allows up to 2×10^7 monovalent cations s^{-1} flow across the membrane, causing a fatal collapse of essential H^+, Na^+ and K^+ concentration gradients between the bacterium and its external environment. Xenobiotics, including many biocides, often are toxic because they disrupt channel protein functions. *Lyngbya* spp. (Cyanobacteria) produce a toxin that interferes with the functioning of an Na^+ channel protein. Puffer fish poison (tetrodotoxin) acts on proteins that transport Ca^{2+} (Li et al. 2001). Ivermectin, a lipophilic antiparasitic drug, used against helminth parasites, kills by causing disruption of channel proteins that govern trans-membrane trafficking of Cl^- ions (Slimko & Lester 2003). Insect resistance to pyrethroids and DDT involves mutations in genes that code for Na^+ transport proteins (Liu et al. 2000, Ranson et al. 2000). In the fungus gnat *Drosophila melanogaster*, resistance to the insecticides dieldrin and fiprinol involves changes to Cl^- channel proteins and can be invoked by a single-point genetic mutation (French-Constant et al. 1993, Housi et al. 1995).

Carrier proteins

Carrier proteins transport materials by chemically binding the substance to the receptor sites. Small bacterial carrier proteins move substances from one surface to the other by diffusion through the lipid bilayer. Large carrier proteins reconfigure before releasing the transported substance on the opposite side of the biological membrane. When substances are transported against their diffusional or electrochemical gradients, the process is powered by ATP (Alberts et al. 1994) (Fig. 4). Some protein types transport single molecules; others function as coupled transporters with different substances shuttled across the membrane simultaneously, sometimes in the same direction, sometimes in opposite directions. A23187, a protein that transports Ca^{2+} and Mg^{2+} into a cell carries 2 H^+ ions out for every divalent cation carried in (Alberts et al. 1994). In the

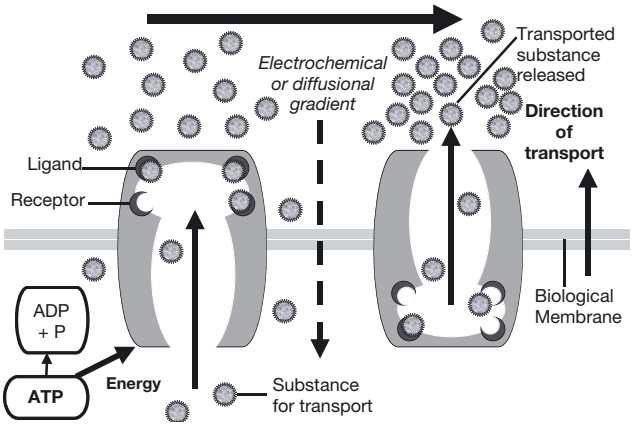

Fig. 4. Diagrammatic representation of carrier protein that utilises ATP to transport a substance against its electrochemical and concentration gradient. (Illustration by S. Quinnell adapted from Alberts et al. 1994 and Voet et al. 1999)

hepatopancreas of the lobster *Homarus americanus*, Cl^- can use 3 coupled transporters (SO_4^{2-}, oxylate^{2-} and HCO_3^-) (Gerencser et al. 1995). The flowering plant *Catharanthus roseus* absorbs the herbicide glyphosate through an Fe transporter, which is stimulated into action by Ca^{2+} (Tilquin et al. 2000). Transport of NO, which is synthesised from L-arginine, is governed by the proteins that transport L-arginine (Closs et al. 2000, Stevens et al. 2000). Transport proteins can be identified through the use of protein inhibitors (Gerencser et al. 1995), a technique common in molecular biology and biochemistry, and one that should be applied in ecotoxicology.

Why pollutants may be imported

From the foregoing it is clear that pollutants can only cross the impermeable biological membrane through protein-regulated admission. Here, 2 adventitious mechanisms are proposed.

Similarity. This mechanism operates when a pollutant's chemical structure is highly similar to that of an essential molecule, allowing the pollutant to form a ligand with carrier proteins. An example is the intracellular transport of CO in vertebrates by its attachment to haemoglobin at the site that normally binds O_2. Because the haeme–CO bond is 210 times stronger than the haeme–O_2 bond, CO tends to displace O_2, causing CO poisoning (Van De Graaff & Fox 1992). Similarly, oestrogen mimicking substances (e.g. many biocides, phthalate esters) may act by binding onto oestrogen receptor proteins.

Concealment. This allows pollutants to cross the biological membrane undetected because they are bound

to large metabolically essential units (Patton 1981, Kanda et al. 1990). Examples include pollutants bound to an organic food molecule (e.g. a fatty acid or polypeptide) (Kanda et al. 1990), or to a molecular complex (e.g. micelles, liposomes and lipopolysaccharides) that is imported. Concealment is likely to be the route by which most pollutants enter metazoans, including those pollutants that are highly insoluble in water (e.g. many organic biocides and industrially synthesised chemicals). Such uptake probably occurs on any external epithelial cell surface. The movement of concealed substances between a cell and its environment was proposed in medicine more than 20 yr ago (e.g. Patton 1981, Kanda et al. 1990), but has received virtually no attention in ecotoxicology.

Pollutant elimination

Ecotoxicology's diffusion model assumes that pollutant elimination also occurs by passive diffusion (e.g. Hawker & Connell 1985, 1988a,b, Connell 1988, Stenerson 1992, Randall et al. 1998). However, an array of proteins capable of excreting many types of xenobiotics, including lipophilic substances, have been identified. The actions of some of these underlie major medical problems, including antibiotic and multi-drug resistance (MDR), as well as biocide resistance in pest and weed species (Alberts et al. 1994, Schäfer et al. 1996, Al-Awqati 1999, Closs et al. 2000, Stevens et al. 2000, Thumser & Storch 2000, Ueda & Matsuk 2000, Chaumont et al. 2001, Garcia et al. 2001, Hu & Wu 2001, Marples 2001, Nejsum et al. 2001, Sansom & Law 2001, Zeuthen et al. 2001, Raggers et al. 2002, Madigan et al. 2003). Ivermectin, a lipophilic drug toxic to helminths, is tolerated by mammals if their cells can rapidly synthesise the appropriate proteins to eliminate the drug (Schinkel et al. 1994, Umbenhauer et al. 1997, Smith & Pritchard 2002). The ubiquitous large ABC superfamily of exporters is well known (van Veen et al. 2000). Increased expression of P-glycoproteins (an ABC subgroup) in insects has been linked to insecticide resistance (Retnakaren et al. 2001), with activity levels of P-glycoprotein-mediated processes related to levels of environmental pollution (Smital et al. 2000).

Detection of a xenobiotic in cells triggers expression of exporter proteins, provided the appropriate genetic code for their expression exists. Possession of pre-existing genetic codes indicates exposure to similar cytotoxins in an ancestral cell/organism and conservation of the genetic response through generations. Pollutants which pose the lowest threat to life probably will belong to classes of molecules encountered frequently during evolutionary history (e.g. hydrocarbons and some heavy metals), allowing opportunity for the evolution of genetic codes to express the appropriate export proteins. The most toxic pollutants should be those that are environmentally novel or rare in biologically available forms as well as industrially synthesised chemicals (e.g. organochlorides, PCBs, dioxin and some heavy metals).

The diffusion model cannot account for observed high rates of cross-membrane trafficking, such as very high metabolic flux rates of virtually insoluble long-chain fatty acids into tissues (Thumser & Storch 2000), nor can it explain disparities in xenobiotic uptake and elimination between organisms within single species, such as why mice lacking the gene for expression of *mdr1a* P-glycoprotein accumulate 100 times more Ivermectin (a lipophilic drug) in their brains than mice with the appropriate gene (Schinkel et al. 1994). The diffusion model also cannot explain the very rapid impact on living organisms of highly lipophilic insecticides, whose solute concentrations and, therefore, rates of aqueous diffusion are very slow. The functioning of transport proteins provides a credible explanation of pollutant uptake and elimination by cells, organelles and organisms, although the process may be an indirect consequence of the proteins' intended biological functions (see later subsection 'Delivery of pollutants to receptor proteins').

Endocytosis–transcytosis–exocytosis cycle

In eukaryotes, transport proteins are located in pits at sites of need (domains) within cells, with sometimes thousands of transporters for a specific chemical often concentrated in a single pit (Alberts et al. 1994). Formation of ligands between the substance to be transported and a critical number of transport proteins in a pit triggers endocytosis, the most conspicuous material-uptake process in the cell. Endocytosis consists of 2 primary forms, phagocytosis and pinocytosis, although intermediate processes are known. Phagocytosis is limited to amoeboid cells, which use it to incorporate large particles and whole microorganisms (Roitt 1994, Lodish et al. 2000), and is apparently not a significant pollution uptake route (Kujawinski et al. 2000). Pinocytosis occurs in virtually every type of eukaryotic cell (Alberts et al. 1994), and is thus the dominant form of endocytosis. In macrophages the entire biological membrane's surface area goes through pinocytotic invagination every 30 min and thus an external substance can be rapidly transported inside the cell (Alberts et al. 1994). The rate of biological membrane in-pocketing is determined by the concentration of the substance for transport in the cell's external environment and the density of carrier proteins on the cell surface (Lodish et al. 2000). Pinocytosis is presumably

how pollutants concealed on ingested material are incorporated into eukaryotic cells.

The continual loss of biological membrane through endocytosis is offset by exocytosis, by which transport proteins eliminate materials from cells (e.g. wastes, signalling-molecules and xenobiotics). Exocytosis occurs at the same rate as endocytosis, ensuring that the total surface area of a cell or organelle is maintained (Alberts et al. 1994). Specific exocytotic protein transporters are located at different sites on the biological membrane according to an organelle's, cell's or tissue's function (Alberts et al. 1994). This allows substances to be excreted from individual cells (Sun et al. 2000) to specific extracellular locations (skin, blood, lymph, nerve synapses, gut lumen, etc.), or excreted (by liver, pancreas, nephridia gills and skin) (Cunningham 1992).

'Transcytosis' here refers to the processing of pollutants within a cell. Besides the process of intracellular digestion (hydrolysis), the nucleus issues instructions for protein synthesis, the Golgi apparatus synthesises proteins, and the ER delivers proteins to where they are needed. Most transcytosis pathways are less well known than transport processes at external cell membranes, but have been shown to involve transport proteins; also sequestering of toxins may occur at the organelle level (Rajagopal & Simon 2003). Breakdowns in transcytosis may be reflected by alterations to physiological processes (Wong et al. 2001).

The endocytosis–transcytosis–exocytosis cycle, as applied to pollutant uptake and elimination from eukaryotic cells (the protein model), is represented in Fig. 5, which postulates the fate of DDT ingested as a food contaminant entering through a simplified gut-wall epithelial cell. Because of its high lipophilicity, DDT is assumed to have attached to intestinal liposomes and micelles of fatty acids (see following subsection), although any lipidic molecules may act as DDT carriers. The process of endocytosis presented here is based on the well-known cholesterol uptake pathway (Alberts et al. 1994, Voet et al. 1999). Carrier proteins responsible for endocytosis cluster in pits on the cell's surface. Endocytosis is activated when sufficient micelles and liposomes in the pit have formed ligands with the substance for transport (Sue et al. 1993, Billingsley & Lahne 1996, Voet et al. 1999). The pit seals off to form an endosome, and commences the process of transcytosis (Alberts et al. 1994). The newly formed endosome migrates inwards and fuses with a lysosome (a sack of hydrolysing enzymes) (Alberts et al. 1994), allowing intracellular digestion to decompose the micelles, liposomes and associated DDT cargo, releasing fatty acid metabolites and metabolites from the decomposition of DDT (e.g. DDE, DDD). All metabolites are then sorted and exported by the appropriate proteins in the vesicle's membrane. Non-hydrolysed DDT, as well as DDE and DDD, will be sequestered within the cell if export proteins are not available for their elimination. However, in this model, we show pollutants being eliminated by an ABC export protein. These eliminated pollutants are transported by proteins within plasma to sites of metabolism (e.g. liver), elimination (e.g. kidney, gills, skin, intestine) or sequestration (e.g. liver, lungs, gills, kidneys). They may also be incorporated into internal micelles and liposomes within the circulatory system (not shown) and thence transported to sites of elimination or sequestration. Successful elimination of any pollutant from a cell or

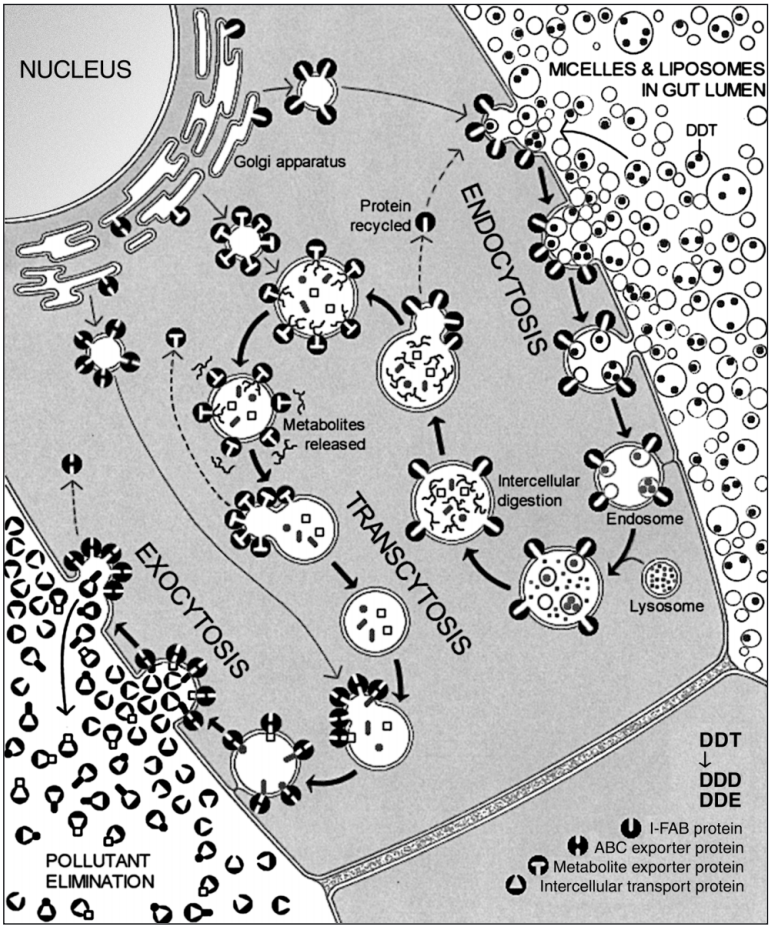

Fig. 5. Protein model of pollutant uptake and elimination. For details see text. (Illustration by Sally Elmer & S. Quinnell)

living organism will depend on the existence of appropriate export proteins. Other biomolecular processes are known to play a role in limiting impacts of pollutants, especially insecticides, by chemically binding to the insecticide, inactivating it, and sequestering it internally (e.g. Kostaropoulos et al. 2001). The function of such sequestering molecules also needs to be examined by ecotoxicology.

Delivery of pollutants to receptor proteins

It has been argued that pollutant diffusion rates in water will ultimately determine cellular uptake rates because pollutants must first diffuse through the external aqueous environment before reaching an organism's transport proteins. This is not so. In aquatic environments (including interstices of soils), micelles and liposomes form spontaneously from phospholipids, and can encapsulate and deliver high concentrations of both lipophilic and hydrophilic materials (Alberts et al. 1994). Micelles and liposomes are highly abundant within the gut lumen and intercellular body fluids and in intercellular fluids (Alberts et al. 1994). Transmembrane crossing of micelles and liposomes is complex and does not involve simple diffusion (e.g. Di Toro et al. 1991, Park et al. 2002)

Micelles are formed from a single-tailed phospholipid layer, and have external hydrophilic heads that interact readily with water and a hydrophobic/lipophilic inner cavity that excludes water (Alberts et al. 1994), but which readily accommodates lipophilic materials (Cserháti et al. 2002, Park et al. 2002) (Fig. 6). Liposomes consist of bitailed phospholipids arranged into 1 or more bilayers that internally encapsulate solutes, but may carry lipophilic materials attached to their lipidic wall (Alberts et al. 1994, Voet et al. 1999) (Fig. 6). Micelles and liposomes composed of fatty acids are readily transported across the biological membrane by forming ligands with the appropriate proteins (e.g. fatty acid-binding proteins: Sue et al. 1993, Billingsley & Lane 1996, Kaufman et al. 2001).

In animals with alimentary tracts, emulsifiers (e.g. bile salts) and dietary fats form intestinal liposomes and micelles (Alberts et al. 1994), which become primary holding vehicles for suspended food. Peristaltic churning of the gut's contents provides opportunities for suspended liposomes and micelles to make contact with appropriate transport proteins (e.g. intestinal fatty acid-binding proteins) in external epithelial cells of the gut wall. This allows uptake of the micelles and liposomes with their cargo (i.e. high concentrations of food molecules) (Sue et al. 1993, Billingsley & Lane 1996). Liposomes are used to carry and deliver soluble and lipophilic drugs (Sue et al. 1993, Alberts et al. 1994). Pollutants bound to micelles and liposomes associated with sediments and soils, as well as those formed in the gut during extracellular digestion of food, probably represent the primary pathway for major dietary uptake of pollutants by deposit-feeding and soil-ingesting organisms. Contaminated micelles and liposomes also pose a threat to infaunal suspension-feeding aquatic organisms, but these are probably exposed to much lower pollutant concentrations than deposit-feeders (see below).

Because micelles and liposomes occur throughout aquatic environments and in soils (Di Toro et al. 1990), they may deliver lipophilic pollutants to other external epithelial cells such as the skin and respiratory surfaces. Solute pollutants as well as lipophilic pollutants (e.g. many hormone-mimicking contaminants) may be encapsulated within naturally occurring liposomes and micelles. It needs to be established if micelles and liposomes encapsulate both lipophilic and hydrophilic pollutants, as this will be crucial to understanding the uptake of organic pollutants and probably heavy metals.

Deposit-feeding infauna also appear to be more vulnerable to pollutants than suspension-feeders because their diet incorporates much larger quantities of bacteria and their exopolymers (Hoskins et al. 2003). Detritus, especially plant matter, is a principal nutrient and energy input into estuarine ecosystems (Odum 1971, Hutchings & Saenger 1987), much of it settling onto sediments (Zabetoglou et al. 2002). However plant matter, especially cell-wall carbohydrates, is not readily accessed by heterotrophs until it has been decomposed by bacteria and fungi. Up to 6.5×10^9 bacteria ml^{-1}

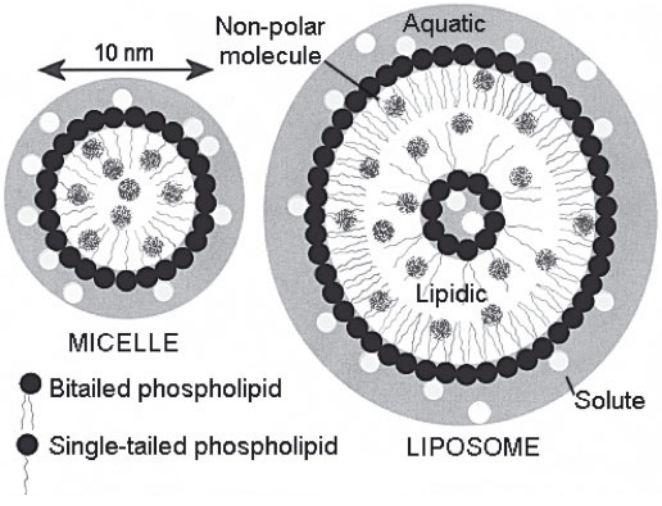

Fig. 6. Micelle and liposome composed of phospholipids showing how their structures confer ability to contain and transport solutes and non-polar lipophilic molecules in aqueous environments. (Illustration S. Quinnell adapted from Alberts et al. 1994 and Voet et al. 1999)

were reported in sediments from Australian estuaries (Hutchings & Saenger 1987). These bacteria form extensive external lipidic biofilms of exopolymers (e.g. lipopolysaccharides of Gram-negative bacteria) (Hoskins et al. 2003, Madigan et al. 2003), which offer binding sites to an array of pollutants, especially organic chemicals. Nutrients released by bacterial and fungal detritus decomposition adsorb onto sediment particles, providing nourishment for larger microorganisms (Fenchel 1970, Madigan et al. 2003). These microorganisms, fungi and bacteria are the primary food of deposit-feeders (Lillebø et al. 1999, Wolff et al. 2000).

Suspension-feeders collect food from the water column (Gili & Coma 1998, Turner & Millward 2002), where microbial population densities are usually lower than in sediments (Madigan et al. 2003). Thus, the primary food of many suspension-feeders is eukaryotic organisms, such as eggs and plankton, although suspension-feeders may also collect microbes (Gili & Coma 1998). Sedimentation/resuspension undoubtedly cycles particle-sorbed pollutants between the sediments and water column, but the concentrations of most pollutants are much higher in sediments than in associated water (e.g. Tagatz et al. 1987, Hamer et al. 1999, Fent 2003, Van der Oost et al. 2003). Thus, deposit-feeders are more exposed than suspension-feeders to most contaminants.

Research in ecotoxicology must begin to include studies of protein-mediated cross-membrane uptake, internal transport and elimination of pollutants, and consideration of the roles micelles and liposomes play in pollutant delivery. Major changes in the monitoring for pollutants in the environment are required urgently, so that we can more realistically assess the actual impact of pollutants on living organisms and ecosystems.

CONCLUSION

The movement of molecules into and out of organelles, cells, tissues and entire organisms is a protein-governed mechanism in both prokaryotes and eukaryotes. Such regulation is essential because all cells are contained within the impermeable biological membrane. Protein-mediated cross-membrane trafficking takes a number of forms, including facilitated diffusion (channel proteins), active transport (carrier proteins) and, in eukaryotes, endocytosis (phagocytosis and pinocytosis), transcytosis and exocytosis. Intracellular and probably intercellular transport also may be mediated by proteins. Transport proteins may act in isolation, cluster in pits or form complexes of many types. They also are located specifically in cells according to their function.

This paper has concentrated on pollutant uptake from the gut lumen of higher animals. However, any epithelial cell (e.g. skin, lungs and gill membranes), may be an uptake site, especially in aquatic ecosystems where liposomes and micelles are abundant in the eternal environment. Plants possess similar regulatory mechanisms on cell walls and membranes (Raven et al. 1992). Following uptake, the fate of incorporated pollutants is determined by an organism's physiology, metabolism and the range of proteins it is genetically coded to produce.

In aquatic ecosystems, pollutants bound to macromolecules (e.g. polypeptides, polysaccharides, liposomes, micelles and bacterial exopolymers) pose a much greater threat to living organisms than do the same pollutants in their solute (pore water) phase. Under the 'concealment' hypothesis presented here, solutes may not be available for uptake. Macromolecules tend to associate with detritus, sinking and accumulating within sediments. Therefore, deposit-feeding infauna are highly vulnerable to sediment-sorbed contaminants. The concentrations of most pollutants to which deposit-feeding benthic infaunal organisms will be exposed most often will be determined by the quantity of those substances sequestered in the substrate, and not by their dissolved aqueous component. Similarly, the concentration of pollutants to which either benthic or pelagic suspension-feeding organisms are exposed will be determined by the concentration of the pollutant bound to organic matter, especially molecular complexes that are food for suspension-feeders, and not by water concentrations. Infaunal suspension-feeders are less vulnerable to pollutants than deposit-feeders because they utilise a different food resource. Notably, deposit-feeding organisms ingest large amounts of microbial biofilms, which provide ideal binding sites for pollutants, whereas suspension-feeders do not.

Within the science of ecotoxicology, ingestion of pollutants sorbed to essential macromolecules is not considered as a pathway by which sediment-sorbed pollutants enter aquatic food webs, nor is the role of micelles and liposomes as transport vehicles emphasised. Both are likely to be critical determinants of pollutant uptake. Therefore, global standards for chemical toxicological testing for contamination of both aquatic sediments and terrestrial soils based on pore water concentrations may be leading to a continual underestimation of the risks posed by many contaminants.

The diffusion model in use in ecotoxicology is irreconcilable with biological understanding of how living organisms regulate their internal environment. The only instance in which pollutants may diffuse passively across the biological membrane is if the cell is dead or

dying. Not surprisingly, it has been noted that it is virtually impossible to predict the fate of xenobiotic substances with simple partitioning models (Van der Oost et al. 2003). The protein model should be applied.

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