Toxicity vs. hormesis in evaluating health effects: applications to bioassays using marine organisms

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An unexplored, yet challenging subject in ecological risk assessment may be ascribed to the impact of aquatic contaminants at subtoxic concentration ranges. Background is provided by the studies of hormesis (or positive stimulation of biological events) in bioassay studies in view of a predictive evaluation of pollutant toxicity to environmental health. When running bioassays, dose- or concentration-related effects imply acute toxicity at the highest tested agent (or mixture) levels, whereas exposures to low agent levels have been associated to either linear or to threshold trends, accordingly regulated in the legislation for xenobiotics and physical agents. Aside to these established criteria, an extensive body of literature has reported that dose- or concentration-related trends for radiation and chemicals show a low-level interval where the adverse effects – observed at higher agent levels – are reverted to a positive stimulation of the biological events being evaluated including, e.g., increases in growth rate, fertilization success, or enzyme activities. This stimulation is currently termed hormesis, and its relevance has been raised in a number of disciplines, such as radiobiology, carcinogenesis, and toxicology/pharmacology. Hormesis has been reported in a number of organisms, in vitro and cell-free systems, also including several bioassays utilizing marine biota, from the pioneering studies by Stebbing (1979) utilizing hydroid coelenterates up to recent papers based on sea urchin bioassays (De Nicola *et al*., 2004; in press). The extent and relevance of the hormesis phenomenon have been reviewed by Calabrese and his co-workers (2005), and a review of the literature in the last 25 years shows a dramatic increase of papers referring to hormesis, from scanty publications until 1995 up to approximately 400 papers cited in MedLine from 2001 to August 2006. The evaluation of concentration-related shifts from hormesis to toxicity requires adequate design in bioassays, including: a) broadly ranging agent concentrations, and b) adequate definition of controls. Concentration intervals should be designed in a realistic range above background contaminant levels, yet below the classical "no-adverse-effect-level" (NOAEL), that may conceal the onset of hormetic effects provided that adequately low agent concentrations are tested. If bioassays are to measure parameters as growth rate, or enzyme activity, untreated controls provide values that may either be increased (hormesis) or decreased (toxicity) as a function of agent levels. Otherwise, a number of parameters may range along with an event frequency, such as 0% to 100%. In this case, assigning optimal values to controls will prevent from assessing any hormetic effect when compared to a "perfect" control value. Thus, bioassays relying on event frequencies should not assign optimal values in control schedules, whereas sub-optimal control values may allow us to assess hormetic effects, if any. Based on the experience of bioassay studies, the relevance of hormesis in the "real world" – e.g. in coastal water and sediment - may be envisioned. Beyond the limited scope of bioassays, the major relevance of

hormesis in environmental health and risk assessment relies on the consequences of exposures to subtoxic levels of contaminants in confined water bodies (e.g. enclosed bays), in dilution ranges of contaminants that may exert hormetic activity. As far as some biota may thrive as an effect of hormesis, the ensuing population burst for the affected biota may result in a set of imbalances at the community level. Thus, targeted field studies might detect possible hormetic effects of low-level contaminants leading to selective changes in population densities for some species with detrimental consequences to other biota.

HORMESIS: DEFINITION AND HISTORICAL BACKGROUND

A growing body of literature in toxicology, pharmacology and radiobiology reports on hormesis, a phenomenon that may be defined as a positive stimulatory effect exerted by low concentrations, or doses, of agents that cause inhibitory or toxic effects when administered at increasing levels. Far from being an extravagant statement, the observed shifts from stimulatory ("hormetic") effects to inhibition and toxicity may be ascribed back to the Ancient Greek term *pharmakon*, with the opposite meanings of «poison» and of «medicinal agent». A first pioneering study by Hugo Schulz (portrayed in Figure 1) in 1888 was conducted to test a theory proposed by Rudolph Arndt, reporting on the effects of several toxicants in yeast cultures, showing that high level-associated toxicity was reverted to growth stimulation when yeast was exposed to low agent levels (Schulz, 1888). Thereafter this phenomenon was also termed "Arndt-Schulz effect", and numerous reports were published in the early decades of the 20th century (reviewed by Calabrese and Baldwin, 2000a,b). A renewed interest in the studies of hormesis has been attributed to the early reports by Anthony Stebbing and his co-workers (1976; 1979; 1982) that opened a re-appraisal and still-thriving investigations on hormesis in environmental sciences and in other disciplines (reviewed by Calabrese, 2005; Calabrese and Blain, 2005). The growing awareness of this phenomenon in the scientific community, however, has failed as yet to result in any consequences in terms of actions by either environmental protection or drug regulatory agencies (van der Schalie and Gentile, 2000; Calabrese, 2001; Kitchin and Drane, 2005; Mundt, 2006).

Fig. 1. Hugo Paul Friedrich Schulz (1853-1932).

HORMESIS: A BROAD RANGING PHENOMENON

Evidence for the growing interest in hormesis is given in Figure 2, showing the steady increase of MedLine-archived publications (updated August 2006) displaying the term "hormesis" in their titles and/or abstracts. Over 730 similar citations were retrieved from Web of Science as compared to the 406 found in PubMed (1956-2006).

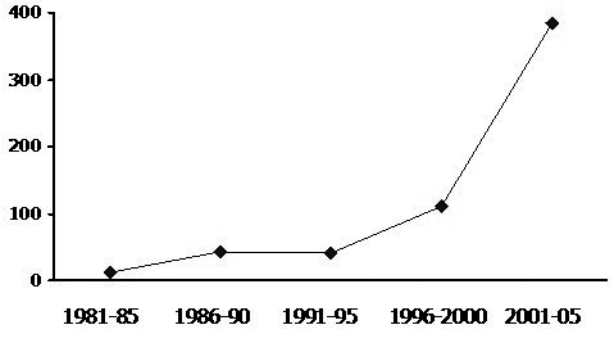

Fig. 2. Production of journal articles including the term "hormesis" as retrieved by MedLine (August 2006).

The literature records include physical agents, inorganics, a diversified range of organics (including pesticides, solvents, pharmaceuticals, and carcinogens), and complex mixtures, as shown in Tables 1a to 1d. The organisms where hormetic effects were assessed include a broad range of phyla, from bacteria and microalgae up to higher plants and mammals. The endpoints evaluated in hormesis studies mostly focused on cell growth, yet a number of other parameters were evaluated, including organismal, cellular and molecular endpoints, such as life span, success in early development, cell differentiation, and protein expression and regulation (Tables 1a, b, c, d).

Table 1a. Selected hormesis-related literature from MedLine (August 2006): physical agents.

Agents	Testing objects	Endpoints	References
Distance from a nuclear power plant	Humans	Blood cell counts	Lee *et al.*, 2001
Gamma irradiation	Bacteria and yeast	Growth rate	Petin *et al.*, 2003
	Paramecium tetraurelia	Growth rate	Croute *et al.*, 1982
	Mice	Life span	Caratero *et al.*, 1998
	Jack pine *(Pinus banksiana)*	Seed germination	Sheppard *et al.*, 1992
X-rays	Mouse hematopoietic progenitor cells	BFU-E, CFU-GM, and c-kit+ cells	Wang and Cai, 2000; Li et al. 2004
	Mouse thymocytes	Expression of RIP-10 protein	Chen *et al.*, 2000
	Rat splenocytes	Con A-induced proliferation	Ishii *et al.*, 1990
	Drosophila melanogaster	Larval lethality and imaginal life span	Vaiserman *et al.*, 2003
Heat shock	*Drosophila melanogaster*	Hsp70 synthesis	Kristensen *et al.*, 2003; Hercus *et al*, 2003
	Caenorhabditis elegans	Life span	Cypser and Johnson, 2003
	Human mesenchymal stem cells	Osteoblast differentiation	Norgaard *et al.*, 2006
Heat, UV, and reactive oxidants	*Caenorhabditis elegans*	Life span	Johnson *et al.*, 2002

Table 1b. Selected hormesis-related literature from MedLine (August 2006): inorganics.

Agents	Testing objects	Endpoints	References
As(V), Cu(II), Cd(II), Cr(VI), Hg(II), Pb(IV), Ti(IV)	Human mammary cells	Viability and cell proliferation	Schmidt *et al.*, 2004
	McCoy cells	Production of heat shock protein 70	Damelin *et al.*, 2000
	Drosophila melanogaster	Courtship, fecundity and motor activity	Hirsch *et al.*, 2003
	Phormia regina	Pupation success	Nascarella *et al.*, 2003
	Lumbricus rubellus	Survival and length of juvenile period	Spurgeon *et al.*, 2004
	Vibrio fischeri	Growth rate and viability	Fulladosa *et al.*, 2005
	Daphnia magna	Wet weight and reproductive rate	Tsui and Wang, 2006
	Avena sativa	Weight, height and chlorophyll content	Kuzel *et al.*, 2003
Organic cupric salts	Honeybees	Population lethality	Bounias *et al.*, 1995
Tributyltin chloride (TBT)	*Crassostrea virginica*	Hemocyte chemiluminescence	Anderson *et al.*, 1997
Inorganic model mixtures [As(V) + Cd(II) + Cr(VI) + Pb(IV)]	Human keratinocytes	Cytotoxicity	Bae *et al.*, 2001

Table 1c. Selected hormesis-related literature from MedLine (August 2006): pesticides and other organics.

Agents	Testing objects	Endpoints	References
2,4-dichlorophenoxyacetic acid; 6 organophosphorous insecticides	Chlamydomonas reinhardtii (mt+)	Growth, photosynthesis and chlorophyll a synthesis	Wong and Chang, 1988
Chlorpyrifos	*Daphnia carinata*	Survival, fecundity, first brood	Zalizniak and Nugegoda, 2006
Permethrin	*Supputius cincticeps*	Ovary activation	Lemos *et al.*, 2005
Enrofloxacin	Plants *(Cucumis sativus, Lactuca sativa, Phaseolus vulgaris, Raphanus sativus)*	Growth of primary root, hypocotyl, cotyledons and leaves	Migliore *et al.*, 2003
Flumequine	Aquatic weed *(Lythrum salicaria)*	Number and size of leaves and secondary roots	Migliore *et al.*, 2000
Lindane	*Bryocamptus zschokkei*	No. eggs and viable offspring	Brown *et al.*, 2003
Ethanol	Chick embryo myoblasts	Content in water-soluble proteins	Matiushichev and Sokolov, 1996
Nonylphenol	*Microcystis aeruginosa*	Growth and microcystin production	Wang *et al.*, 2006
Alpha-benzene hexachloride	Rats	GST-P foci, preneoplastic lesions	Puatanachokchai *et al.*, 2006
Nitroaromatic compounds	Terrestrial plants *(Medicago sativa, Lolium perenne, Echinochloa crusgalli)*	Seedling emergence, fresh shoot, and dry mass	Rocheleau *et al.*, 2006
Solvents (15 compounds)	Soybean seedlings	Upregulation of TIP/NOX protein	Morre, 1998

Table 1d. Selected hormesis-related literature from MedLine (August 2006): miscellaneous.

Agents	Testing objects	Endpoints	References
Pharmaceuticals			
21 pharmaceuticals	Fish cell lines	Mitochondrial MTT reduction and neutral red uptake	Caminada *et al.*, 2006
Phenobarbital	Rats	Hepatocarcinogenesis	Kinoshita *et al.*, 2003
Complex mixtures			
Mixture of 6 substances	Algal communities	Carbon fixation	Backhaus *et al.*, 2004
Extracts from seaweeds	Fungi *(Colletotrichum gloeosporioides* and *Rhizoctonia solani)*	Growth rate	Barreto *et al.*, 2002
Spiked sediment with 3 explosives (TNT, RDX and HMX)	*Chironomus tentans* and *Hyalella azteca*	Survival	Steevens *et al.*, 2002

Most of this literature is confined to testing individual agents by single species bioassays. However, efforts have been devoted to gain information on hormetic effect by testing model or complex mixtures in multi-species studies, as e.g. in the report by Backhaus *et al*. (2004) who tested the effects of mixtures in algal communities. Studies at the microcosm or at the ecosystem level are not reported, to the best of our knowledge, and await appropriate design and implementation.

HORMETIC EFFECTS IN MARINE ORGANISMS

Hormetic effects have been reported in marine bacteria, yeast, micro- and macroalgae, coelenterates, polychaetes, bivalves, echinoderms, and fishes, as shown in Table 2. The agents displaying hormetic effects in marine biota include inorganics, metallorganic compounds (TBT), and several organics as pharmaceuticals and complex mixtures. The recorded endpoints included growth rate, reproductive and developmental success, and tissue regeneration (Table 2). Chemiluminescence methods have been utilized, e.g. hemocyte activation in bivalves, and luminol-dependent chemiluminescence in sea urchin gastrulae, as shown in Figure 3 (Pagano *et al*., 2001a,b).

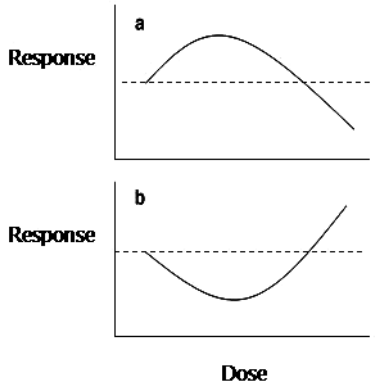

Fig. 3. The most common form of the hormetic dose-response curve depicting low-dose stimulatory and high-dose inhibitory responses. A. the β- or inverted U-shaped curve. B. the hormetic dose-response curve depicting low-dose reduction and high-dose enhancement of adverse effects, the J- or U-shaped curve (from Calabrese, 2004).

Table 2. Bioassay reports on hormesis utilizing marine organisms.

Biota	Agents	Endpoints	References
Marine bacteria *(Vibrio fischeri)*	Cd(II)	growth rate and viability	Fulladosa *et al.*, 2005
Marine yeast *(Rhodotorula rubra)*	Cu(II)	growth rate	Stebbing, 1987
Marine algae *(Nitzschia closterium; Platymonas subcordiformis; Dunaliella tertiolecta)*	anthrancene; streptomycin	growth rate	Huang *et al.*, 2002
	tannin mixtures; tannery wastewater	growth rate	De Nicola *et al.*, 2004; 2006
Hydroid Celenterates *(Laomedea flexuosa) (Campanularia flexuosa)*	Cu(II)	growth rate	Stebbing, 1976; 1982; 1987; Moore and Stebbing, 1976
Polychaetes *(Hydroides elegans)*	Cu(II), Al(III), Pb(IV), Ni(II), Zn(II)	fertilization and embryogenesis	Gopalakrishnan *et al.*, 2006
Echinoids *(Paracentrotus lividus, Sphaerechinus granularis)*	Cd(II); As(III); As(V); Arochlor 1254; mitomycin C; diepoxybytane; tannin mixtures; tannery wastewater	fertilization; embryogenesis; mitotic activity; chemiluminescence	Pagano *et al.*, 1982a,b; 1986; 2001 Trieff *et al.*, 1988; Korkina *et al.*, 2000; De Nicola *et al.*, 2004; 2006
Bivalves *(Mya arenaria; Mya truncata; Mytilus edulis; Siliqua costata)*	$AgNO_3$; $CdCl_2$; CH_3HgCl; $HgCl_2$; $ZnCl_2$	hemocyte phagocytosis	Sauve *et al.*, 2002
Crassostrea virginica	Tributyltin chloride (TBT)	hemocyte chemiluminescence	Anderson *et al.*, 1997
Fish *(Fundulus heteroclitus)*	Cd(II)	fin regeneration	Weis & Weis 1986

An example of hormesis in marine organisms exerted by complex mixture may be provided by our recent studies of vegetable and synthetic tannins utilized in leather tanning industry, and of tannery wastewater in sea urchin *(Paracentrotus lividus* and *Sphaerechinus granularis)* early

development and in growth rate of marine and freshwater microalgae *(Dunaliella tertiolecta* and *Selenastrum capricornutum)*. Individual tannin compounds were reported to display a hormesis to inhibition shift, as e.g. tannic acid and gallic acid, as shown in Table 3. In our studies, vegetable tannin from *Acacia* sp., synthetic phenol-based tannin, and leather tannery wastewater shared hormetic effects at comparable tannin concentrations. As shown in Figure 4, fertilization rate of sea urchin sperm displayed an increase vs. controls at low tannin levels. The same applied to the frequencies of developmental defects in tannin-exposed sea urchin embryos, as shown in Figure 5. As discussed below, the hormesis/toxicity shift reached statistical significance provided that control quality was set at suboptimal frequencies of developmental defects, whereas the assignment of optimal control quality either displayed a similar, yet non-significant trend, or turned to monotonic concentration-related trends.

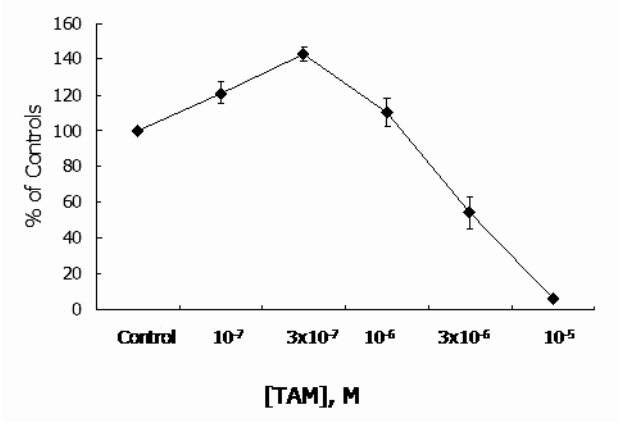

Fig. 4. Luminol-dependent chemiluminescence in sea urchin *(Paracentrotus lividus)* gastrulae exposed to tamoxifen (from Pagano *et al.,* 2001a).

Table 3. Selected information on tannin-related toxic and hormetic effects.

Agents	Effects	References
Camellin B	induced apoptosis in HeLa cell line	Wang *et al.,* 2001
Hypericum perforatum extract & oil	↑ immunostimulating activity in rats ↑ immunosuppressing activity	Anonymous, 2001
Gallic acid	non-toxic up to 5 g/kg body weight in mice	Rajalakshmi *et al.*, 2001
Areca nut polyphenols and tannin	oral cancer promotion	Jeng *et al.*, 2001
Terminalia arjuna tannin extract	↓ 2AF –induced mutagenicity	Kaur *et al.,* 2000
Tannic acid	↑ metabolic activation of a few mutagens anticlastogenic and antimutagenic effects	Chen and Chung, 2000 Sasaki *et al.,* 1990
Tannins	↑ inhibitory activity on lipid peroxidation	Hong *et al.,* 1995

PLANNING HORMESIS-ORIENTED STUDIES

Concentration range

In most bioassay reports, agent concentrations, or doses range from NOAEL to acute toxicity or lethal effects. Within this range, two types of trends are commonly recognized, i.e.: a) threshold trends, characterized by a critical concentration/dose superimposable with NOAEL and above which adverse effects are detected, and b) linear trends, assumed by most agencies for radiation, genotoxins and carcinogens, deemed to exert decreasing, yet definite damage even at very low-level exposures.

In the presence of these established criteria, hormetic effects may arise from serendipitous observations and, even so, may be disregarded as "data fluctuations" below threshold or at very low agent levels. Additional factors in failure to recognize hormesis may be attributed to a widespread unawareness of this phenomenon, and/or to a form of discomfort to report an *improvement* of an adverse event for low levels of an agent exerting damages when administered at higher levels.

Beyond this widespread attitude and practice, focusing studies on possible hormetic effects both requires open-minded workers and, especially, demands a study design that shall encompass a broad concentration range that must include low agent levels, e.g., for merely practical purposes, by three to five orders of magnitude below threshold (Calabrese and Blain, 2005).

Also advised is to plan suitable numbers of subjects in animal studies for low-level exposures fitting the requirement to improve the statistical power of data, as suggested by Hunt (2002).

A crucial issue: control quality

Whenever the bioassay endpoint is quantified by e.g. measuring enzyme activities, growth rate or any other number that may be attributed to "normal", or control values, then inhibition and, respectively, hormesis will be associated with lower or higher values compared to controls. On the other hand, defining suitable controls in detecting hormetic effects may be viewed as a *sine qua non* in all those bioassays whose endpoints are measured by relative frequencies, e.g. expressed by decimal or percent values. In these bioassays, a broadly shared prejudice claims that controls should be characterized by 100% of a physiological event (e.g. fertilization, or hatching success), and by zero value for adverse events (e.g. malformations or other abnormalities). This criterium, by definition, hides any hormetic effect, as no improvement of performance can be detected vs. controls, once control quality has been established up to optimal values. In the case of fertilization success, establishing control values at 100% may conceal an additional pitfall, as 100% fertilization may actually hide a theoretical (and impossible) higher-than-optimal fertilization rate due to excess sperm cells compared to the available ovocytes. Hence, in this case one is both unable to assess any hormetic effect (fertilization rate cannot exceed 100%) and a loss of test sensitivity also occurs vs. inhibitory effects, as spermiotoxicity may only be detected once excess sperm are inactivated. In this kind of bioassay, control quality should be defined *below* optimal values, e.g. by setting control values at values closer to a "central" performance (e.g. 50 to 70% for physiological events), thus allowing for the observation of both adverse outcomes – without any sensitivity bias – and by enabling the observation of hormetic effects. As shown in Figure 5, fertilization rate of sea urchin sperm was either enhanced or inhibited provided that fertilization rate was established at suboptimal values.

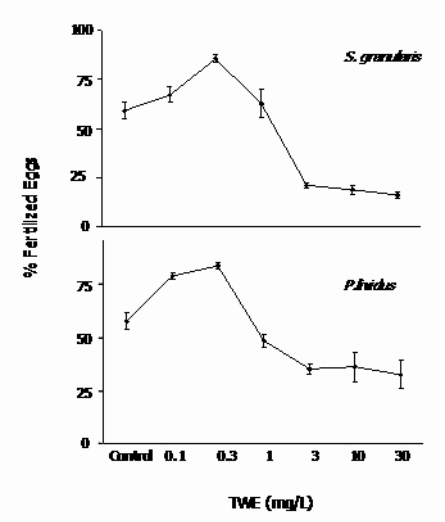

Fig. 5. Fertilization rate of sea urchin *(Paracentrotus lividus and Sphaerechinus granularis)* sperm exposed to tannin water extract (TWE) (from De Nicola *et al.,* 2004).

As an additional, yet not neglectable argument, one might note that optimal control quality may, or may not reflect the actual situation in natural populations, where the occurrence of adverse events may be higher than zero and physiological events may be far from a 100% performance.

Therefore, both on the basis of experimental evidence and of common sense considerations (of mathematical and of ecological nature), one may recommend to avoid relying on unrealistic and illogical criteria as those attributing an optimal performance to control cultures.

RELEVANCE OF HORMESIS AMONG ENVIRONMENTAL HEALTH EFFECTS

The occurrence – or the relevance - of hormesis has been debated stating that this phenomenon cannot be generalized nor can be utilized as a default assumption in toxicology (Kitchin and Drane, 2005). A recent Staff Paper issued by the US EPA (March 2004) stated that as "the purpose of a risk assessment is to identify risk (harm, adverse effect, etc.), effects that appear to be adaptive, non-adverse, or beneficial may not be mentioned", as discussed by Mundt (2006).

Several arguments may be raised against this attitude (Calabrese, 2004). Hormesis is currently associated with the onset of "beneficial" effects, a definition that has mostly derived from studies of individual agents tested in single-organism bioassays. However, this definition may disregard some effects of hormesis as they may especially apply to aquatic ecosystems. In spite of the current scarcity of reports on hormesis in community studies (Backhaus *et al*., 2004) and in multi-species bioassays (Barreto *et al*., 2002; De Nicola *et al*., 2004; in press; Huang *et al*., 2002; Migliore *et al*., 2003; Steevens *et al*., 2002) one may anticipate that hormetic effects may be detected at the community level, provided that *ad hoc* studies are carried out. In other terms, if bioassay reports assessing the toxic effects of xenobiotics were later confirmed in exposed communities, one may wonder why the hormetic effects of some defined xenobiotics should not be observed in microcosms or at the community level. Provided that hormetic effects, at a given agent level, may involve some components of the community, the outcome may imply excess population growth for some species at the expenses of other biota, in analogy with the well-established effects of excess nutrient levels (Occhipinti-Ambrogi *et al*., 2005). Hence, a "non-adverse, or beneficial" effect for some species may result in overall perturbations of often fragile community balances.

Another aspect of possible adverse effects induced by low-level toxicants may be exemplified by the fertilization success of sea urchin (*Echinus esculentus*) sperm exposed to cadmium or zinc salts administered separately or in equimolar mixtures (Pagano *et al*., 1986). As shown in Figure 6, a shift from hormesis to inhibition of fertilization success was exhibited by each of the agents in the micromolar range, whereas a dramatic spermiotoxic effect was exerted by the model mixture of Cd(II) + Zn(II) in the same concentration range. This early result may point to an as yet poorly explored subject, i.e. concentration-dependent trends by model mixtures and, even more so, by complex mixtures of agents that exert hormesis/toxicity shifts when administered individually.

Together, background laboratory experience should prompt new investigations focused on the potential adverse effects of hormesis – especially by complex mixtures - at the ecosystem level.

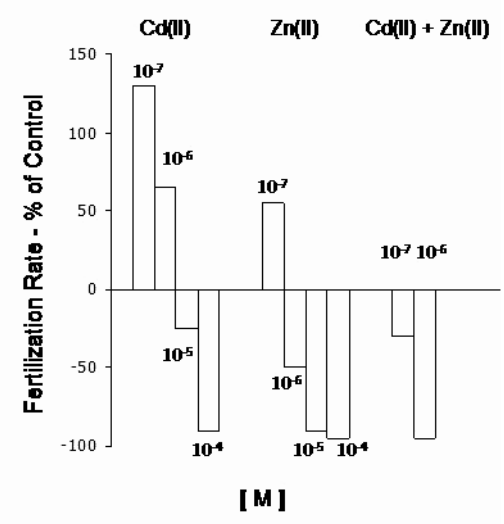

Fig. 6. Hormesis/inhibition shift in fertilization success of *Echinus esculentus* sperm exposed to $CdCl_2$, $ZnCl_2$, or their equimolar mixture (from Pagano *et al.,* 1986).

PROSPECTS IN ECOLOGICAL RISK ASSESSMENT

As stated by Chapman (2001), "a major research need is the extension of hormesis beyond chemical stressors to abiotic (e.g., habitat) and biotic stressors (e.g., species introductions, organism interactions). An overreaching research need is to determine for all stressors with model organisms, populations, and communities whether hormesis has positive, neutral, or adverse effects". To date, the phenomenon called hormesis has been broadly documented, yet confined to bioassay studies. Some opinions point to hormesis as a generalized phenomenon, resulting in "stimulatory" or "beneficial" effects (Calabrese, 2004); yet we still ignore to what extent individual xenobiotics or mixtures do – or do not – exert hormetic effects, provided that adequate studies are designed. Even less we know about the extent low-level agents exert beneficial, no, or adverse effects at the ecosystem scale. The answers to these open questions are most likely diversified according to the xenobiotics or mixtures to be investigated. However, even in the case of a real advantage taken by low-level agents in a community, this advantage may be reasonably confined to some biota and not to others, hence resulting in uncontrolled alterations in the ecosystem. Novel research avenues are open in elucidating these amazing environmental problems.