Regenerative Response and Endocrine Disrupters in Crinoid Echinoderms: An Old Experimental Model, a New Ecotoxicological Test

M.D. Candia Carnevali

Abstract The regenerative phenomena that reproduce developmental processes in adult organisms and are regulated by endocrine and neurohumoral mechanisms can provide new sensitive tests for monitoring the effects of exposure to anthropogenic chemicals such as *endocrine disrupter* (ED) contaminants. These pollutants in fact can be bioaccumulated by the organisms, causing dysfunctions in steroid hormone production/metabolism and activities and inducing dramatic effects on reproductive competence, development and growth in many animals, man included. Current research is exploring the effects of exposure to different classes of compounds well known for their ED activity, such as polychlorinated biphenyls (PCBs), nonylphenols and organotins, on regenerative potential of echinoderms, a relatively unexplored and promising applied approach which offers the unique chance to study physiological developmental processes in adult animals. The selected test species is the crinoid *Antedon mediterranea*, which represents a valuable experimental model for investigation into the regenerative process from the macroscopic to the molecular level. The present study employs an integrated approach which combines exposure experiments, chemical analysis and biological analysis utilizing classical methods of light (LM) and electron (TEM and SEM) microscopy and immunocytochemistry. The experiments were carried out on experimentally induced arm regenerations in controlled conditions with exposure concentrations comparable to those of moderately polluted coastal zones in order to reproduce common conditions of exposure to environmental contaminants. The results of the exposure tests were analysed in terms of effects at the whole organism, at the tissue and cellular level, and possible sites of action of EDs. Our results show that prolonged

M.D. Candia Carnevali (e-mail: daniela.candia@unimi.it)
Dipartimento di Biologia, Universita' degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

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exposure to these compounds significantly affects the regenerative mechanisms by inducing appreciable anomalies in terms of regeneration times, overall growth, general morphology and histological and cellular pattern. A concentration/effect relationship could be found for all the substances. Interestingly, contrasting results in terms of inhibition or acceleration of regeneration phenomenon were obtained for the different chemicals.

1 Regeneration and Its Biological Implications

Development in animals does not necessarily mean embryogenesis and does not always start from an egg, fertilized or not. Regeneration is in fact a distinct type of developmental process typically occurring in adult animals: it can involve limited processes of cell turnover and tissue repair, replacement of lost parts or organs, and even complete regrowth of whole individuals from small body fragments. Due to its obvious close relation with fission phenomena and cloning processes, regeneration can be considered the specific developmental strategy complementary to asexual reproduction, in the same way as embryogenesis is complementary to sexual gametic reproduction. Therefore, although regeneration unavoidably involves analogous problems of cellular identity and positioning and often superficially resembles embryogenesis in an accelerated form, this basic difference in its intrinsic asexual starting-point makes regeneration a significantly different biological process. In embryogenesis, the structure is totally created ex novo, whereas in regeneration an already existing structure is reformed after its loss or severe injury and the new cells develop in an established context of mature tissues and cells. Therefore, in all animals, the regenerative processes related to different organs and structures should be regarded as fundamentally distinct developmental processes rather than as an accelerated recapitulation of ontogenetic processes (Candia Carnevali and Bonasoro 2001a).

Although a response to injury is evoked in all animals, the degree of morphological and functional recovery can show a remarkable variability not only between unrelated groups, but also between closely related species. In contrast to the old traditional views which regard the regenerative potential as a prerogative of the simplest and most primitive animals, regeneration is actually a widespread phenomenon through phylogeny and its quite heterogeneous distribution from the lowest to the highest phyla appears to be independent of their organization and complexity level (Ferretti and Géraudie 1998; Thouveny and Tassava 1998; Candia Carnevali and Bonasoro 2001a). In fact, the regenerative capabilities appear to depend upon the individual potential for histogenetic and morphogenetic plasticity expressed in terms of recruitment of stem cells and/or dedifferentiated cells, cell proliferation and migration, supply of specific regulatory/trophic factors, and finally expression or re-expression of the developmental programme in adult animals.

1.1 Regeneration in Echinoderms

In spite of the wide choice of potential models for studying regeneration, this phenomenon has been extensively explored only in a few animals, which traditionally monopolized the attention of the developmental biologists. In contrast, with regard to many animal groups well known for their regenerative capabilities, there are surprising gaps in knowledge in terms of not only cellular and molecular aspects of regeneration, but also basic mechanisms (Candia Carnevali and Bonasoro 2001a).

Regarding general mechanisms, regeneration in all animals is traditionally considered to involve one or other of two basic processes, epimorphosis and morphallaxis. In epimorphosis, new tissues arise from undifferentiated cells (stem cells or dedifferentiated cells) which form a typical blastema. This is a discrete centre of proliferative activity providing a pool of new cells which can give rise to all the regenerated structures. In morphallaxis, extensive phenomena of rearrangement/recycling from existing tissues take place: no blastema is involved and there is only limited and localized proliferation of cells derived from existing tissues by dedifferentiation, transdifferentiation and/or migration. In spite of this apparently clear and well-established difference between epimorphosis and morphallaxis, recent results obtained in the same model in different experimental conditions (see crinoid arm regeneration, Candia Carnevali and Bonasoro 2001b) suggest that the traditional classification of these two processes is too reductive and that the mechanisms at the tissue/cellular level can be largely interchangeable and interpretable in a more plastic and dynamic light.

Echinoderms, which thanks to their spectacular regenerative capabilities were the favourite models of the pioneer *regenerationists* of the 19th and early 20th centuries, after a long period of unexplainable neglect, were reproposed to our attention by a series of recent papers (for review see Candia Carnevali and Bonasoro 2001b; Candia Carnevali et al. 2001 c; Thorndyke and Candia Carnevali 2001) exploring the basic mechanisms of the regenerative phenomenon and its cellular and molecular aspects. Regenerative potential finds in echinoderms its maximum expression (Hyman 1955). It is a common phenomenon in all the classes, extensively employed to reconstruct external parts (arms or other appendages) and internal organs (gonads, gut, visceral mass) often subjected to amputation, self-induced or traumatic, rapidly followed by complete successful regrowth of the lost parts. Regeneration in echinoderms is largely a predicted phenomenon and in most cases follows autotomy, which can be considered the most important proximate cause of structural loss and depends on the presence and properties of "mutable collagenous tissues" (MCTs) (for a review see Wilkie 2001; Wilkie, this Vol.) at the level of the autotomy plane. Under physiological conditions regeneration is prompted by autotomy and proceeds from the retained side of a fractured autotomy plane.

Reconstitutive regeneration of arms is particularly frequent in crinoids and ophiuroids which have fragile arms often involved in self-induced or traumatic mutilations. Interestingly, in many cases, the detached body fragments can survive in good health for a long time and undergo phenomena of partial or total regeneration independently of the *donor* animal (Candia Carnevali et al. 1998). These phenomena, which are also quite common in asteroids, provide evidence of the wide exploitation and implications of regenerative potential in echinoderms. In particular in asteroids, besides the extensive application in common repair mechanisms, arm regeneration offers in fact the most complete example of cloning strategies. As well known, in a few starfish species, individual autotomized arms can regenerate to produce new complete adults. This extreme case clearly shows that in echinoderms regeneration is an indispensable complement of the programme of asexual reproduction which leads to the development of new individuals through fission mechanisms (Emson and Wilkie 1980; Mladenov and Burke 1994). Besides asteroids, also many ophiuroids and holothuroids undergo asexual propagation involving the splitting of adults into two or three pieces, with subsequent regenerative development of complete individuals from each isolated portion. This extensive and strategic employment of regenerative phenomena throughout the phylum indicates that in echinoderms regeneration actually represents an essential component of the life cycle, and this has a wide range of biological implications. In fact, if a close correlation between the regenerative potential of the individual and its possibility of survival can be inferred easily, self-repair abilities not only appear to be an undoubted advantage for the individual, but also give a fundamental contribution to the adaptive capacities of the species and its fitness, since they increase the individual's chances of reproducing, sexually or asexually, even when it is dramatically compromised in its body integrity.

Regeneration by both epimorphosis and morphallaxis is found in echinoderms (Candia Carnevali and Bonasoro 2001a). Epimorphosis with blastema formation seems to typically occur whenever regeneration is a widely predictable, rapid and effective phenomenon, which takes place following autotomy (for instance in crinoids and ophiuroids; Candia Carnevali and Bonasoro 2001b; Thorndyke et al. 2001). These epimorphic mechanisms appear to reproduce rather closely what usually happens in embryonic developmental processes. In contrast, morphallaxis seems to be a more complicated and slower regenerative process which tends to follow traumatic mutilations, for instance in arm tip regeneration of asteroids (Mladenov et al. 1989; Moss et al. 1998). In this case, amputation is not a predictable event and the morphogenetic mechanisms imply phenomena of substantial rearrangement of the old structures, which appear to be unique to regeneration and not shared by embryonic development. As stated above, investigation of crinoid regeneration under different experimental conditions (see Candia Carnevali and Bonasoro 2001b) suggests that this distinction is quite artificial, and that the borders between these two processes

are not so defined, especially in terms of mechanisms at the tissue/cellular level.

Although occurring in all echinoderm classes, regeneration has been studied most thoroughly in crinoids, ophiuroids and asteroids (for review see Candia Carnevali and Bonasoro 2001b; Candia Carnevali et al. 2001 c; Thorndyke and Candia Carnevali 2001). In holothuroids it is also ubiquitous but has been explored in terms of mechanisms only in a few cases (Dolmatov and Ginanova 2001; Garcia-Arraras and Greenberg 2001). Regeneration also occurs in echinoids, but is limited in terms of extent and degree of capabilities and only a few examples have been investigated (Dubois and Ameye 2001; Bonasoro et al. 2004). In the species examined so far most results throw light on aspects related to wound healing, growth, morphogenesis and differentiation, but in most cases many crucial questions remain largely unanswered, especially those related to specific cellular and molecular aspects, and much work is still required. In fact, also in echinoderm regeneration, a complete interpretation of the real biological significance and evolutionary implications of the phenomenon can be achieved only by a broad-spectrum integrated understanding which emerges from diverse perspectives and from different experimental approaches applied to the most representative models.

1.2 The Regenerative Potential of Crinoids

Crinoids are well known for their spectacular regenerative capabilities extensively and successfully employed to reconstruct both external parts, namely arms, pinnules and cirri, and internal organs, such as digestive apparatus, gonads and even complete visceral mass, which can be frequently lost following traumatic injury, predation or spontaneous autotomy. Specimens collected in nature always show regenerating arms at different stages of growth (Fig. 1a). These regenerative phenomena can be easily reproduced in the laboratory by mimicking the autotomy conditions and amputating the arms at the level of the autotomy plane (sutures) (Fig. 1b–d). Arm regeneration in crinoids represents the most thoroughly explored model in echinoderm regeneration studies (Candia Carnevali and Bonasoro 2001b). Recently, we have carried out a comprehensive study of the overall process of arm regeneration in the comatulid *Antedon mediterranea*, a valuable and flexible experimental model previously successfully employed by old classical studies of developmental biology (Perrier 1873; Minckert 1905; Reichensperger 1912), which was re-explored in all its aspects from the macroscopic to the molecular level.

This phenomenon can be described on the whole as a typical blastemal regeneration in which new structures develop from migratory pluripotential, actively proliferating cells in the presence of presumptive regulatory factors. The overall process can be subdivided into three main phases (Fig. 2a): a repair phase, an early regenerative phase and an advanced regenerative phase,

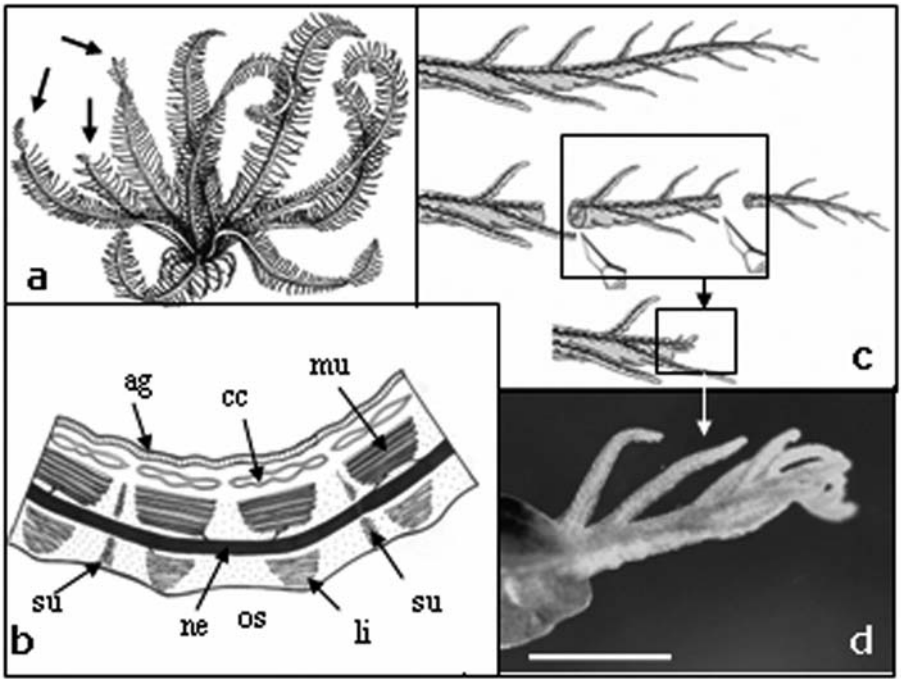


Fig. 1. Schematic presentation of the experimental model. **a** Specimen of *Antedon mediterranea* with three regenerating arms at different stages (*arrows*). **b** Main anatomical features of the arm in sagittal section. *ag* Ambulacral groove; *cc* coelomic canal; *li* ligament; *mu* muscle; *os* ossicle; *su* suture. **c** Normal arm, experimental amputations and regenerating arm. **d** Stereomicroscopic view of regenerating arm (2 weeks post-amputation). *Bar* 1 mm

whose crucial aspects are related to common fundamental mechanisms such as (1) intervention of stem cells and/or employment of dedifferentiated cells (Fig. 2 b–e), (2) cell migration and proliferation, (3) contribution of putative growth factors, particularly in terms of specific neurally derived factors and (4) mechanisms of pattern formation. The data obtained so far are derived from an integrated approach which utilizes different methods (first of all classical methods of microscopy – LM, confocal, TEM, SEM – and specific methods of immunocytochemistry, but also basic methods of biochemistry and molecular biology) on experimentally induced arm regenerations (standard or abnormal) obtained under significantly different experimental conditions, including extreme mutilations (*explants*) or exposure to specific types of environmental contaminants. In particular, the normal mechanisms and pattern of the regenerative processes under standard conditions have been established in serial experiments of regeneration at different stages following pseudo-autotomic amputations (Candia Carnevali et al. 1993, 1995, 1997). A parallel analysis has been carried out on the regenerative processes of both the normal regenerating arms and the respective amputated arm segments

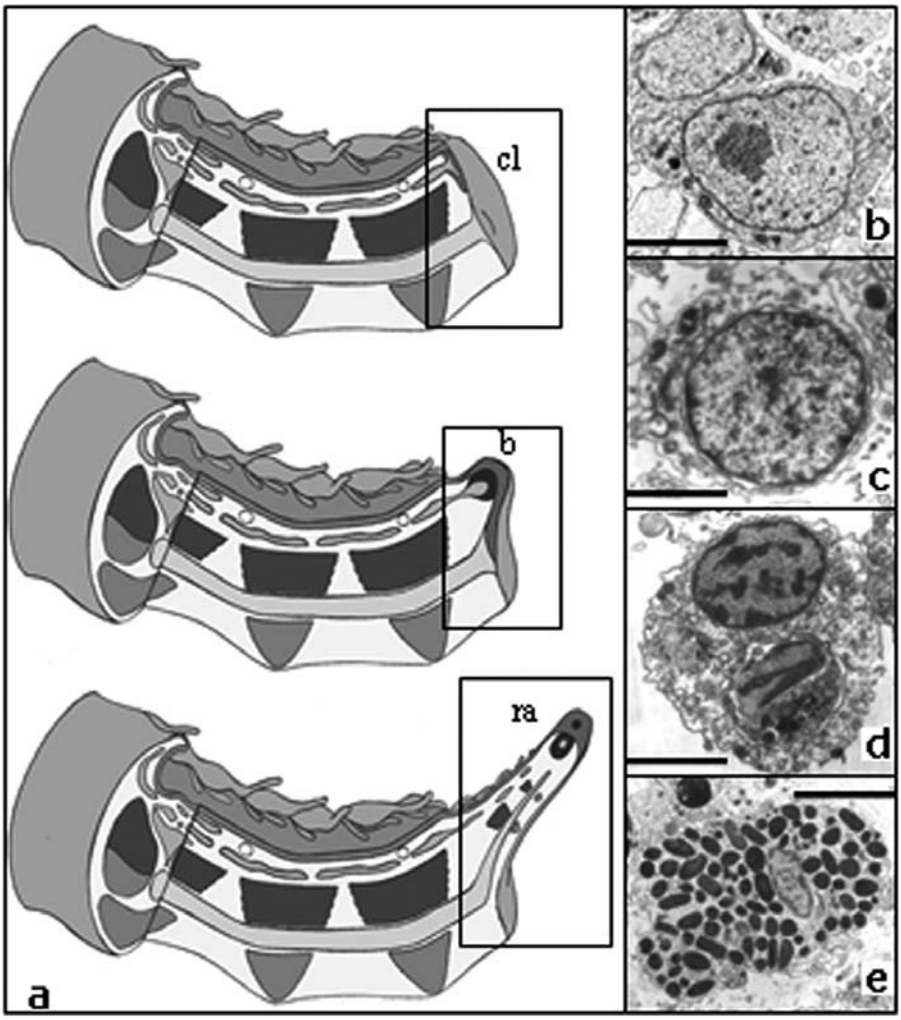


Fig. 2. a Schematic reconstruction of the main phases of arm regeneration. *Top downwards* Repair phase [0–24 h post-amputation (pa)], early regenerative phase (24–72 h pa), advanced regenerative phase (72 h to 3 weeks pa). *b* Regenerative blastema; *cl* cicatricial layer; *ra*: regrowing arm. **b–e** TEM: migratory cells involved in regenerative processes. Presumptive stem cells: amoebocyte (**b**), coelomocyte (**c**), phagocyte (**d**), granulocyte (**e**). *Bars* 4 μm

(*explants*, Fig. 1 c; Candia Carnevali et al. 1998), which can be maintained under good living conditions for about 3 weeks and represent excellent models for testing the arm regenerative potential in terms of autonomy of resources and control and for comparing regenerative mechanisms in the same individual. Different types of isolated explants have been successfully employed: during the culture period they are able to undergo extensive repair and regenerative processes in parallel with their donor arms. Comparison

between the regenerative processes of arm explants and normal regenerating arms of corresponding stages highlights that beside general similarities in the basic regenerative processes there are some meaningful differences in terms of mechanisms employed and cellular/tissue elements involved. The regenerative potential, mechanisms and pattern have also been explored and compared under other experimental conditions, particularly with regard to aberrant regenerations resulting from arms deliberately subjected to traumatic mutilations which do not reproduce autotomy (Candia Carnevali and Bonasoro 2001b). The bulk of the results obtained so far in crinoids not only throw light on the most relevant aspects related to wound healing, morphogenesis, differentiation and growth in echinoderm regeneration, but also strongly suggest employing this fascinating and promising experimental model for a successful applied approach.

2
Endocrine Disrupters and Echinoderms

Some persistent and widely diffused contaminants of anthropogenic origin can be easily bioaccumulated by the organisms and exert their effects as "*endocrine disrupters*" (EDs). According to a recent definition (Holmes et al. 1997; Stahl et al. 1999), an ED is "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function". This means that these compounds can affect significantly the exposed organisms, by modifying the homeostatic status of hormones, particularly steroids, mimicking the action of natural hormones, interfering synergistically or antagonistically with their synthesis, activity and metabolism, interacting with their nuclear receptors and, finally, inducing dramatic effects on gene expression (Colborn et al. 1993; Soto et al. 1995; Fairley et al. 1996; Gray et al. 1996; Cooper and Kavlock 1997; Cadbury 1998).

There is a long list of compounds (including pesticides, fungicides, insecticides, commercial and industrial chemicals, medical drugs, contraceptives) in some cases well known for their adverse effects on organisms, which exert their action by modifying or modulating natural hormonal activity in humans and wildlife populations: this list includes compounds with established estrogenic/anti-estrogenic activity, as well as chemicals with androgenic/anti-androgenic activity. Detailed knowledge of routes of uptake, and pattern of bioactivation, biotransformation and excretion is far from being available for most of these compounds. Another unresolved problem is the equivocal relationship between hormonal disturbances and toxicity. Endocrine dysfunctions, in fact, can be in some cases a secondary response related to toxicity rather than an independent primary response to the chemical (Depledge and Billinghurst 1999). Several wildlife species have demonstrated a significant susceptibility to endocrine-disrupting compounds. In some extensively explored animal models, the exposure to EDs results in

appreciable disruption of steroid hormone production accompanied by related effects on reproductive and developmental mechanisms which can vary a great deal according to the species. Since there are many categories of pollutants displaying these potential actions, it is particularly difficult to find a direct correlation between the exposure levels of the pollutant and its observable effects on organisms, and we are still far from a broad detailed understanding of their potential effects in terms of endocrine-regulated processes. This is particularly true with invertebrates. In fact, in spite of the increasing number of standardized laboratory tests with invertebrate species, there is still a great demand for bioassay systems and/or biomarkers specific for evaluating the potential threats of EDs to invertebrates, which actually represent more than 95 % of the extant animal species in natural ecosystems. It is important to underline that, among invertebrates, there are relevant differences in terms of physiology and biochemistry and the same process can be regulated by different mechanisms of endocrine controls. So, the use of the same biomarker in one taxon has limited value in predicting similar effects in other taxa. Even when a common mechanism is involved in regulating a physiological process among taxa, sufficient differences among organisms with respect to the mechanism itself may significantly alter the level of susceptibility to EDs. Another crucial point in exploring the effects of EDs in invertebrates is related to their mode of action which changes with the seasonal cycle and/or the life stage of the target animal. In invertebrates, in general, ecotoxicological testing has been mainly addressed to measuring adverse effects on early developmental stages, with particular reference to larval stages (Schweitzer et al. 1997; Novelli et al. 2002), which are more sensitive than adults, or to identifying specific biomarkers of a number of relevant endocrine systems in adult organisms.

In recent years, interest has grown in using aquatic invertebrates as tools for monitoring environmental hazards. Although acute and chronic toxicity tests with benthic freshwater organisms are being developed for many compounds, the list of representative organisms for the marine environment is still very incomplete. In spite of the number and variety of potentially useful invertebrate models, and the structural and functional key role of such animals in the marine ecosystem, the attention tends always to focus on vertebrates, even though several excellent examples of ED effects on marine invertebrates are actually available in the literature (for a review see Depledge and Billinghurst 1999). It is enough to quote the phenomena of *imposex* and *intersex* described in gastropod molluscs, which can be regarded as some of the most dramatic and well-documented examples of adverse effects of ED contaminants in the marine field (Bryan et al. 1986; Gibbs et al. 1988; Horiguchi et al. 1995). However, with regard to important marine invertebrates such as echinoderms, the available information is still rather limited, particularly as far as adults are concerned (Kobahashi 1984; den Besten et al. 1989; 1990; 1991a,b; Anderson et al. 1994; den Besten 1998; Coteur et al. 2001; Békri and Pelletier 2004). In fact, the teratogenic effects of various chemical agents and drugs on the development of

embryos and larvae have been slightly more extensively studied, namely in sea urchins (Schweitzer et al. 1997; Novelli et al. 2002). For a review on the effects of physical and chemical pollutants on fertilization and embryogenesis see also Angelini et al., and Matranga et al., this book.

Echinoderms are prime candidates for being selected as marine target macroinvertebrates and utilized as test animals because of their ubiquitous distribution, tractability and sensitivity. They offer a wide range of models for studying the effect of exposure to ED contaminants. A series of important aspects make echinoderms particularly relevant and amenable for this ecotoxicological approach. First, echinoderms are benthic animals and are particularly susceptible to the presence of micropollutants stored in marine sediments. Primary uptake across external epithelia (respiratory surfaces, epidermis, etc.) or secondary uptake from food represent important routes of entry for many dissolved aquatic pollutants which can be rapidly bioaccumulated by these organisms.

Second, regulatory factors and hormones, including peptides and steroids, similar to those of vertebrates have been recently detected and characterized in echinoderms. In particular, considerable published evidence indicates that vertebrate-type steroids can be synthesized (both androgens and estrogens) and used as terminal hormones along the neuro-endocrine cascades regulating reproductive, growth and developmental processes (Schoenmakers 1979, 1980; Schoenmakers and Voogt 1980; Voogt et al. 1984; 1990; 1991; Shirai and Walker 1988; den Besten et al. 1989; Aminin et al. 1995; Shubina et al. 1997; LeBlanc et al. 1999), and they have been demonstrated to control reproductive activities and growth at cellular and tissue level. Current research is actually focusing on echinoderm endocrinology and the improved knowledge in terms of comparative physiology and biochemistry and specific mechanisms involved, including steroid metabolism and possible modulations by ED compounds, is providing a rather good background of necessary information (Janer et al. 2004; Lutz et al. 2004). It is relevant to point out that although individual components of endocrine systems have undergone significant evolutionary divergence in response to specific adaptations, endocrine control strategies and basic hormonal regulatory mechanisms have been, on the whole, rather conserved among closely related animal groups. In terms of evolutionary relationships, echinoderms are deuterostome invertebrates and are phylogenetically more related to chordates than to other invertebrate groups. A short analysis of the phylogenetic distance among genes is given in the chapter by Zito et al., in this book. This means that there is less divergence between echinoderms and vertebrates than between echinoderms and the major protostome invertebrates (annelids, arthropods, molluscs): it is not surprising, therefore, that echinoderms possess control mechanisms of physiological processes rather similar, and maybe nearly homologous, to those of vertebrates, in terms of molecules and actions. In this light, echinoderms may therefore share with vertebrates similar targets in terms of EDs and be susceptible to the same chemicals known for causing reproductive dysfunctions

in vertebrates. It is worth noting that in some echinoderm classes there is limited but significant published evidence of the disruptive effects of contaminants on steroid metabolism and steroid levels and on the effects on the mono-oxygenase (MO) system (den Besten et al. 1989, 1990, 1991a,b; Schweitzer et al. 1997; den Besten 1998). For this reason, echinoderms can be considered key organisms in both basic and applied research in this field and can be usefully employed for developing new successful experimental approaches and strategies.

A third noteworthy point is that many echinoderm species are ecologically relevant and occupy important positions in the food chain of vertebrate wildlife: their loss could have dramatic consequences on the marine ecosystem. In addition, some echinoderm species are edible and are highly valued by humans, whereas other species are relevant for commercial and recreational purposes. So it is important to understand the factors that might influence long-term population viability of these valued resources and to develop a testing programme, in the field and in the laboratory, in order to protect ecologically and economically important species from the potential effects of this dangerous class of environmental contaminants.

A last important characteristic is that echinoderms, as already seen, besides the normal processes of sexual reproduction, have spectacular and unique capacity for regeneration and offer a wide range of models for studying this phenomenon. Regenerating echinoderms can be regarded as particularly valuable experimental models to test the effects of exposure to different types of EDs. In fact, the regenerative phenomena which, as explained above, have the peculiarity of representing developmental processes in the adult organism, are characterized by enhanced and active phenomena of cell proliferation, morphogenesis, differentiation and tissue renewal. They are typically modulated by endocrine and neurohumoral mechanisms comparable to, if not the same as, those usually involved in reproductive and developmental processes. Vertebrate-type regulatory factors, including peptides and steroids, which have been demonstrated to play a role in these processes, are very likely to modulate cellular differentiation and tissue growth during regeneration processes (Candia Carnevali et al. 2001 c; Thorndyke and Candia Carnevali 2001). For this reason, and on the basis of what has been suggested by a few previous data (Walsh et al. 1986; Fingerman 1997), it was assumed that regenerative processes can be susceptible to EDs present in the environment. It is very probable, in fact, that exposure to pseudo-hormonal contaminants can induce variations, in terms of time, mechanisms and actions, in the physiology of regenerative development which is amongst the most sensitive phenomena with respect to environmental stress and consists of stages that can be directly and conveniently monitored for cellular damage. This idea is in agreement with the general trend of ecotoxicological testing with EDs, which has been frequently addressed to detect early effects in developmental stages, embryonic or larval, more sensitive than adults from this point of view. Toxicity tests on the sea urchin *Paracentrotus lividus*, for instance, highlighted that

ED compounds such as organotins can cause critical and consistent damage in early life stages (Schweitzer et al. 1997; Novelli et al. 2002). On the other hand, previous data obtained from the ophiuroid *Ophioderma brevispina* (Walsh et al. 1986) showed clearly that exposure to organotin compounds significantly affected arm regeneration processes, demonstrating successfully how useful the study of regenerative development in adult organisms can be. In the light of what has been seen above, regenerating echinoderms appear to represent ideal bioindicators of ED-induced stress at the whole-organism, cellular and molecular level.

It is relevant to recall that an important goal in studying EDs is not only determining the specific disruptive activity of each compound, but also establishing the most sensitive test species and the most specific forms of response (endpoints) at which the hormonal dysfunction is unequivocally expressed. For this reason, unique endocrine-regulated processes, such as echinoderm regeneration, can provide an important target for toxic action and an original and easily quantifiable endpoint that makes the regenerating animal a very sensitive bioindicator: in fact, it can provide at the same time precious indications of the specific effects of these persistent pollutants on biological mechanisms at the tissue and cellular level and an early assessment of possible degenerative modifications of the marine ecosystem.

3
Crinoid Regeneration and Endocrine Disrupters

On the basis of their spectacular regenerative capabilities, crinoid echinoderms are prime candidates for this ecotoxicological approach. In particular, as previously explained, in the common feather star, *A. mediterranea*, arm regeneration is a common phenomenon which can be easily studied under laboratory conditions thanks to the favourable possibility of inducing experimentally arm autotomy and subsequent regeneration (Fig. 1 c,d), and following the complete regrowth of the lost arm in a relatively short period of time (about 3 weeks). *A. mediterranea* is a widespread species in the Mediterranean Sea, representative of the benthic fauna and easily found. It is a microsuspension-feeding organism on which persistent sediment-bound pollutants have an immediate impact: it can, in fact, concentrate persistent and hydrophobic pollutants directly by primary uptake from water and from suspended matter and sediment, and secondarily from food, thus giving rise to significant biomagnification phenomena. These animals can be maintained in the laboratory for many months and, due to their remarkable capacity for survival, are particularly amenable to the experimental approach. For all these reasons, *A. mediterranea* should be considered as a suitable ecotoxicological model to test endocrine-disrupting activity of selected compounds on a typical marine species. This idea has been recently confirmed by a series of preliminary results obtained with specimens exposed to specific contami-

nants belonging to the ED class, which have clearly shown a wide range of interesting potential effects of the exposure to these pollutants on the regenerative processes at all levels, macroscopic and microscopic (Candia Carnevali et al. 2001a,b, 2003).

In this chapter, we analyse and compare the effects of exposure to different classes of ED compounds on the process of arm regeneration in *A. mediterranea*. The selected types of test compounds are: polychlorinated biphenyls (PCBs), nonylphenols (4-NP) and tripheniltin (TPT-Cl) (Fig. 3). PCBs are commercial products widely used as heat transfer fluids, hydraulic fluids, flame retardants and dielectric fluids because of their unique properties, including resistance to biological and chemical oxidation and nonflammability. Nonylphenols are products of the slow and incomplete biodegradation of nonylphenol polyethoxylates which are widely employed for domestic and industrial uses. TPT-Cl is a typical organotin compound, extensively used in agriculture and in antifouling paints, which tends to accumulate in the soil and sediments.

The widespread use of these different compounds coupled with improper disposal has led to significant global environmental contamination. Although acute and chronic toxicity tests with benthic freshwater animals have being developed for these contaminants, they have to be still definitely established for marine organisms. These three classes of chemicals have widely documented endocrine-disrupting activities which can be expressed by contrasting effects on the exposed animal models. The first two com-

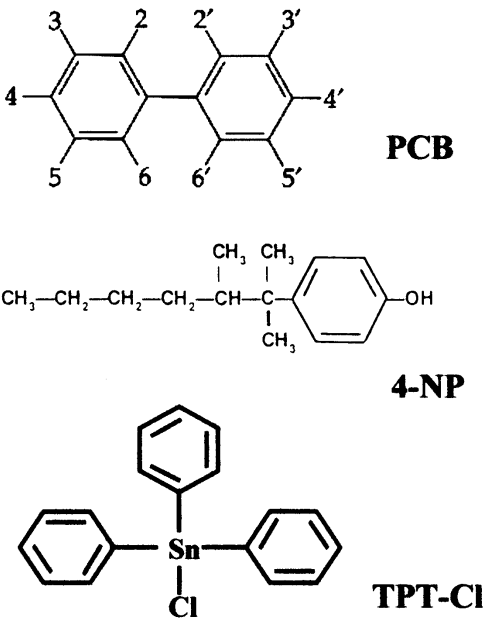


Fig. 3. The tested molecules: typical *endocrine disrupter* compounds

pounds (PCBs, 4-NP) are included in the category of contaminant with estrogenic effects (anti-estrogenic or estrogenic, respectively) (Granmo 1991; Arnold et al. 1996; Dickey 1997; Depledge and Billinghurst 1999;); the third (TPT-Cl) is a typical compound with androgenic effect (Fait et al. 1994; Matthiessen and Gibbs 1998). The aim of this study is (1) to offer a comparative account of the possible insidious effects of exposure to ED contaminants on developmental physiology of typical marine animals, with particular reference to mechanism(s) of action of EDs on growth, differentiation, and repair/regeneration processes at the whole organism, tissue and cellular level in the model species, and to possible homeostatic mechanisms through which the organism can interact and adapt to adverse environmental conditions; and (2) to assess the validity of the regenerative response for bioassay. A relevant point is that in our experiments, exposure to EDs was performed in seawater at concentration ranges reproducing those of moderately polluted coastal areas. The idea was to simulate realistic exposure conditions for wildlife marine fauna.

3.1 Experimental Approach

Our experimental approach employed laboratory tests with individual compounds based on exposure experiments with regenerating crinoid samples. During the overall exposure period, lasting 2 weeks, the concentrations of the selected compounds were measured in the exposure water and in the animal tissues in order to follow bioconcentration kinetics.

3.1.1 Exposure Experiments

Specimens of *A. mediterranea*, collected from along the Tyrrhenian coast of Italy (Giglio Island), were maintained in closed glass aquaria containing 50 l of artificial seawater with an internal circulation system, at 14 °C, and fed with InverteMin (Tetra Marin). Exposure to the selected pollutants was performed under controlled conditions, in terms of environmental parameters and contamination levels, under static or semistatic conditions (20 % water renewal in 24 h) as explained below. The exposure concentration was selected on the basis of available data on real contamination levels in the coastal areas. Groups of 30 specimens were employed in each aquarium (exposure, control and solvent-control aquaria). In each exposed or control specimen, experimental regeneration was induced in two to three arms, mimicking the autotomy conditions. Immediately after the amputation, the experimental animals were put in the test aquaria and exposed to different concentrations of the selected compound for prefixed periods (72 h, 1 week and 2 weeks): in this

way, the exposure period corresponded to well-defined and established regenerative stages. At each stage, chemical analyses of water and echinoderm tissues were performed in order to check the variability of exposure concentration and the bioaccumulation.

3.1.2 PCB Exposure

The exposure to PCBs tests were performed under static conditions by employing a commercial mixture of Aroclor 1260. Some replicates of the experiments were performed at different exposure concentrations. The exposure medium was obtained by adding concentrated water solutions of PCBs (mother solutions) to the exposure aquaria (Candia Carnevali et al. 2001a,b), reaching initial concentrations of 23, 77 and 81 ng/l respectively.

3.1.3 4-NP Exposure

The exposure to 4-NP tests were performed under static conditions. Some replicates of the experiments were performed at different exposure concentrations (2, 5 and 10 μg/l), which were obtained by adding suitable small aliquots (less than 2 ml) of 4-NP acetone solutions in the exposure aquarium until the pre-established final concentration was reached (Candia Carnevali et al. 2003).

3.1.4 TPT-Cl Exposure

The exposure tests to TPT-Cl (Merck) were performed under semistatic conditions (20 % water renewal in 24 h). The experimental animals were exposed to different concentrations of TPT-Cl (50, 100 and 225 ng/l). The exposure medium was obtained by adding to the aquaria 1.25 ml ethanol-TPT-Cl solution at the start of the experiment and 0.250 ml ethanol-TPT-Cl solutions day by day for the 20 % renewal (Barbaglio et al. 2004). The final ethanol concentration in exposure aquaria was 0.025 ml/l (lower than that officially allowed in long-term ecotoxicity tests with aquatic invertebrates). As far as the selected TPT-Cl exposure concentrations are concerned, the maximum concentration was close to LC_{50} experimental values quoted in the literature (Rippen 1990), whereas the minimum concentration was close to NOEC (no observed effect concentration) experimental values known for echinoderms (Walsh et al. 1986).

3.2 Biological Analysis

At whole-organism level the analysis focused on the effects of ED pollutants on crinoid regenerative processes by taking into account abnormal morphological aspects related to growth and development. This analysis was suitably associated with an accurate histopathological investigation focusing on specific anomalies at tissue and cellular levels. This integrated analysis provided extensive information on the possible alterations, resulting in a highly sensitive condition index related to ecologically relevant, individual-level responses. The histological examination, in particular, still provides one of the most rapid and sensitive methods of detecting adverse acute or chronic effects of exposure which represent intermediate levels of response between those detectable at the whole-organism level and those at the molecular level, and can reflect prior toxicant-induced molecular and biochemical aspects of cell physiological alteration (Hinton 1997). Thus the biological aspects of the regenerative processes were investigated in regenerating crinoids at whole-organism, tissue and cellular levels by employing classical morphological methods, both macroscopic and microscopic (stereo, light, electron – TEM, SEM – and confocal microscopy) as described in detail in previous papers (Candia Carnevali et al. 1993; Candia Carnevali and Bonasoro 2001b), associated with specific immunocytochemical protocols and/or statistical analyses whenever appropriate.

Exposed and control regenerating arms were prefixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer for 4–5 h, and then, after overnight washing in the same buffer, post-fixed with 1 % osmium tetroxide in the same buffer. After standard dehydration in an ethanol series, the samples were embedded in Epon-Araldite 812. The semithin and thin sections, cut with a Reichert Ultracut E (diamond knife), were stained by conventional methods (crystal violet-basic fuchsin for LM; uranyl acetate and lead citrate for TEM) and then observed in a Jenaval light microscope and Jeol 100 SX electron microscope respectively. A range of specific immunocytochemistry (ICC) techniques utilizing commercial monoclonal antibodies was employed to monitor cell proliferation (BrdU method; see Candia Carnevali et al. 1995, 1997) or pattern distribution of common cellular biomarkers (cytochrome P450; see Candia Carnevali et al. 2001a,b 2003). The results of the exposure tests were compared with those obtained by a parallel analysis of normal regenerating samples under standard conditions.

3.3 Chemical Analysis: Summary of Analytical Procedures and Results

The chemical analysis performed in parallel with the biological analysis is an important component of the integrated ecotoxicological approach presented

here and the results add great value to those derived from the biological analysis. Nevertheless, the details of the analytical work are rather far from the aims of the present review and, for completeness, it seems to be sufficient to give only a schematic overview of the main points and to briefly summarize the most relevant results obtained so far. Analytical techniques used in quantification include high performance liquid chromatography (HPLC), high resolution capillary gas chromatography (GC) with flame ionization detector (FID) and selective detection, and mass spectrometric (MS) detection. Aquarium water samples were extracted by liquid–liquid extraction or solid-phase extraction according to the polarity of the investigated pollutants.

3.3.1 PCBs

Three exposure concentrations were tested: 23, 77 and 81 ng/l. The concentration in the exposure aquaria was controlled daily during 2 weeks of experiments by extracting the PCB solution in seawater with n-hexane (pesticide grade). The PCB congener concentration decreases with the exposure time: in terms of total PCBs, at the end of the experiments, the analytical values of exposure water were about 20 % of the initial nominal concentrations. For PCB determination in the animal tissues, three animals were collected from each exposure and control aquarium at 72 h, 1 week and 2 weeks. The concentration of chemicals in water and animal tissue extracts was measured by GC-ECD for PCB (Candia Carnevali et al. 2001a,b) after clean-up on a Florisil column (4×0.7 cm i.d.). As far as bioconcentration is concerned, in the tests with a nominal exposure concentration of 77 ng/l the 14-day total PCB concentration was 2,257 ng/g (on a lipid basis), not far from those measured in other filter feeders collected along the Mediterranean coasts.

3.3.2 4-NP

Three exposure concentrations have been tested: 2, 5 and 10 μg/l. The water concentration was controlled at the end of the exposure period by extracting the seawater 4-NP solution with an SPE (solid phase extraction) procedure. A LiChrolut EN cartridge was used as solid-phase column and 500 ml of sample was passed through the column to extract 4-NP which was then recovered with 5 ml of methanol. The 4-NP concentration decreases in the exposure medium with the exposure time: at the end of the experiments the analytical values of exposure water were about 50 % of the initial nominal concentrations. For 4-NP determination in the animal tissues three animals were collected from each exposure and control aquarium at 72 h, 1 week and 2 weeks. HPLC-fluorescence was used to analyse both water and tissues extracts (Can-

dia Carnevali et al. 2003). As far as bioconcentration is concerned, this was quite rapid, the tissue concentration reaching high values (0.4–0.6 μg/g) after a short exposure period (72 h) with the lowest exposure concentration (2 μg/l).

3.3.3 TPT-Cl

Three exposure concentrations were tested: 50, 100 and 225 ng/l. TPT-Cl analyses were performed by gas-chromatographic separation and mass-spectrometry detection after derivatization of the original compound (TPT-Cl) in the extraction medium. In terms of chemical parameters, a detailed chemical analysis of water and tissue samples from our exposure experiments is still in progress (Dagnac et al., unpubl.). Nevertheless, preliminary analytical results clearly indicate that (1) actual concentrations measured in exposure medium are much lower than the nominal ones (i.e. 1.3 and 9.3 ng/l for nominal exposure concentrations of 50 and 225 ng/l respectively; Tremolada et al. 2004); and (2) TPT-Cl concentration in the animal tissues appears to be significantly high (53 ng/g fresh wt.) with the lowest exposure concentration (50 ng/l), in spite of the low final concentrations in the exposure water.

4 Exposure Effects of EDs and Biological Implications on Regeneration

Arm regeneration in *Antedon* consists of a typical epimorphic blastemal process which has been reconstructed in its main phases – repair phase, early regenerative phase and advanced phase – particularly with respect to its cellular and molecular aspects (Candia Carnevali et al. 2001b, 2003). These phases are schematically shown in Fig. 2a in relation to the basic anatomy of the arm, whose main components are detailed in Fig. 1b: a segmental series of brachial ossicles, connected by muscles and ligaments, a central brachial nerve, a multiple system of coelomic canals, and an ambulacral epithelium. Our recent studies have also shown that arm regeneration is a typical nerve-dependent phenomenon in which the nervous system acts as a primary source of regulatory factors involved not only in the regenerative processes of the neural tissue itself, but also to a large extent in development and regrowth of all other structures. In addition, the nervous system and the coelomic canals act as important sources/vehicles for the different types of migratory cells, which are responsible for the regenerative processes, including presumptive stem elements (undifferentiated amoebocytes and coelomocytes), phagocytes and granulocytes (Fig. 2b–e). It should be noted here that coelomic fluids of echinoderms contain different types of cells, generically called coelomocytes, which corre-

spond to structurally and functionally distinct elements. Due to their capability to respond to injuries, host invasion and cytotoxic agents, coelomocytes are regarded as the immune effectors of echinoderms (Chia and Xing 1996; Gross et al. 1999). For a review on coelomocytes see Matranga et al., in this book. Coelomocytes are not yet well characterized functionally in crinoids, but are morphologically distinguishable as undifferentiated (presumptive stem cells, Fig. 2b,c) and differentiated (phagocytes and granulocytes, Fig. 2d,e) cells. These latter cells are apparently typical of crinoids and are extensively employed in repair processes (Fig. 2e). They are characterized by a massive content of dense granules and represent well-differentiated migratory elements, present in the coelomic fluids or scattered in the connective tissues, particularly around the brachial nerve.

In our experiments, the effects of exposure to ED compounds on the regeneration process have been monitored at three regenerative stages, 72 h, 1 week and 2 weeks post-amputation, which are considered significant stages in terms of developmental processes (growth, morphogenesis and differentiation). Here, we give an account of our recent results on the effects of the exposure to different contaminants representative of distinct classes of ED compounds on crinoid regeneration, with particular reference to the following points:

- effects at whole-organism level (mortality, growth, malformations);
- effects at tissue and cellular level (histological pattern, morphogenesis, differentiation);
- possible sites of action, particularly in terms of steroid dysfunction.

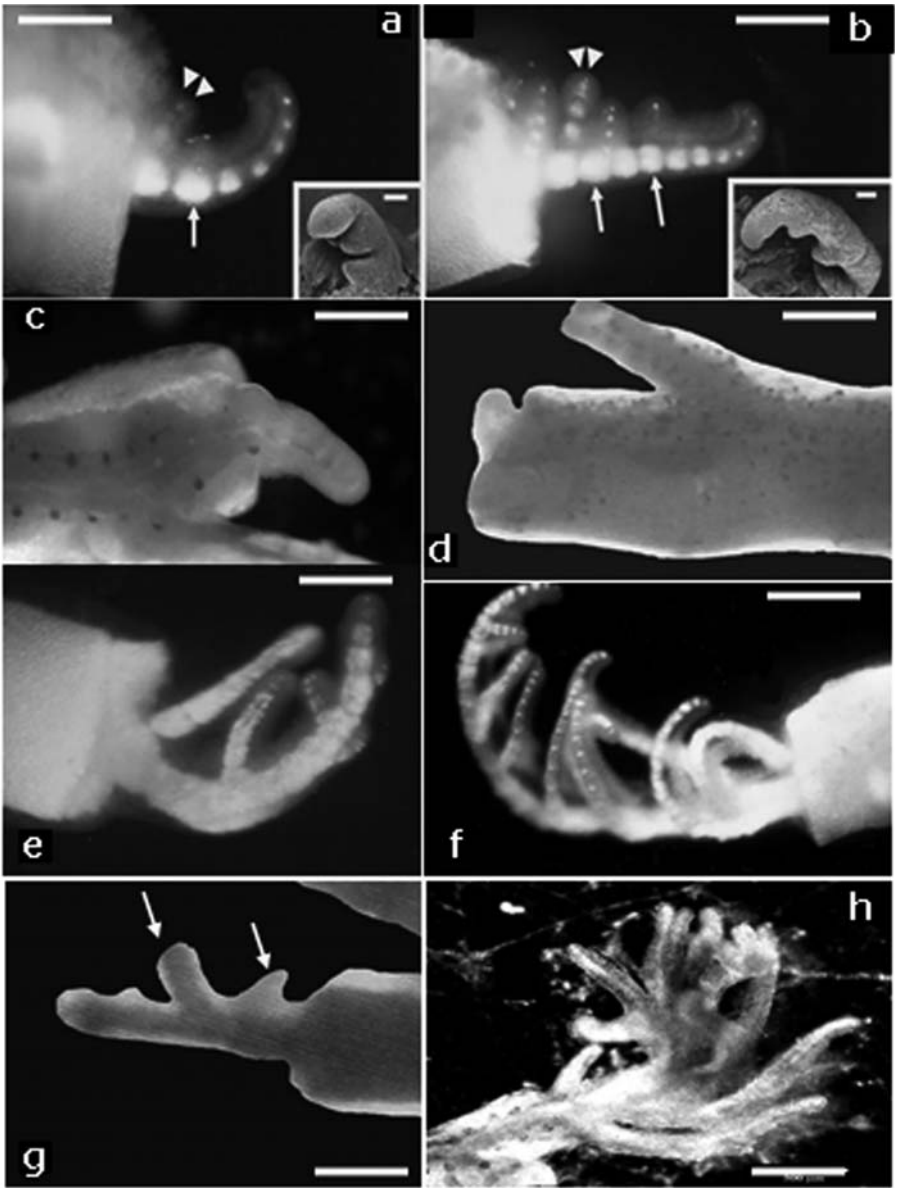
4.1 Mortality

No toxic effects were observed in PCB-exposed animals in the sets of experiments: all the experimental animals survived in good condition until the end of the prefixed exposure period (72 h, 1 week, 2 weeks). In contrast, some mortality was observed for 4-NP at the highest exposure concentration (5 and 10 μg/l), particularly for the longest exposure periods (1 week and 2 weeks). In particular, a mean mortality of 30 % was recorded for 5 μg/l exposure concentration and 60 % for the 10 μg/l concentration. As far as TPT-Cl is concerned, according to the data obtained so far (Barbaglio et al. 2004), no toxic effects were observed with the concentration range from 50–225 ng/l. In all these experiments, the mortality was less than 20 % in both exposed and control samples, a value that is considered acceptable for the validity of the ecotoxicological results. The TPT-Cl concentration of 1,000 ng/l appeared to be close to acute toxicity threshold for *A. mediterranea*; 50 % mortality was evident in specimens exposed to this concentration within the first 72 h (LC_{50}), and, in the surviving samples, growth did not progress further and regenerative development appeared to stop at a very early stage (24–72 h post-amputation).

4.2 Growth

The overall growth of the regenerating arms of *A. mediterranea* was significantly affected by PCBs, 4-NP and TPT-Cl exposure in comparison with control animals (Candia Carnevali et al. 2001a,b; 2003; Barbaglio et al. 2004). However, contrasting results were obtained for the three classes of chemicals: in fact, some compounds accelerated the regenerative growth, whereas others appreciably inhibited the regrowth process. As far as PCB exposure is concerned, if during the early regenerative phase (72 h post-amputation) the exposed samples did not show significant anomalies in terms of growth and development, at the advanced regenerative phase (1–2 weeks post-amputation) an unusual accelerated growth was clearly evident in all the exposed arms in comparison with the growth of the standard regenerating arms at the same stages (Fig. 4a,b,e,f). This involved the overall size of the regenerating arm, which appeared much more developed, and the differentiation of its anatomical structures, external or internal, such as the lateral pinnules and the brachial skeletal components (ossicles). In contrast, 4-NP appeared to cause an appreciable delay in the overall growth of the regenerating blastema observable with all the exposure concentration tested (Fig. 4a,d,e,g). With regard to TPT-Cl-exposed samples, a clear effect in terms of enhanced growth, even though less marked than that of PCB samples, could be also detected (Fig. 4a,c,e,h). A quantitative analysis carried out in parallel with the qualitative morphological analysis on the measured lengths of all the experimental regenerates, in both exposed and control samples, showed that in terms of average overall sizes of the regenerating arms, a clear correlation between growth and exposure could be observed for all the test compounds: the differences in the overall growth were particularly evident at 2-week post-amputation; at this stage, which corresponded to a long-term exposure period, the exposed samples also displayed a remarkable variability in terms of overall growth if compared with the uniform standard size of the controls. Quantitative data showed that in PCB-exposed samples the growth increase was significant at both 1 week and 2 weeks post-amputation (Candia Carnevali et al. 2001a,b), whereas in TPT-Cl-exposed samples a significant effect in terms of enhanced growth could be detected only at the advanced stage of 2 weeks and with the higher concentration employed (225 ng/l) (Barbaglio et al. 2004).

Fig. 4a–h. Whole mount stereomicroscopic views of control and exposed regenerating arms of *Antedon mediterranea* at different stages. Exposure concentrations: PCB: 14 ng/l; 4-NP: 2 µg/l; TPT-Cl: 100 or 225 ng/l. **a–d** 1 week pa (post-amputation): **a** control sample; **b** PCB-exposed sample; **c** TPT-Cl-exposed sample; **d** 4-NP-exposed sample. In the PCB-



exposed sample, abnormal growth of the regenerate and advanced development of its anatomical features are evident. In the TPT-Cl- and 4-NP-exposed samples, delayed and anomalous regrowth is evident. *Arrow* Brachial ossicles; *double arrowheads* pinnules. *Insets* show details of the respective regenerating arms from SEM. **a** and **b**, *bars* 240 μm; *inset bars* 100 μm. **e–h** Regenerating arms at 2 weeks pa: **e** control sample; **f** PCB-exposed sample; **g** 4-NP-exposed sample; **h** TPT-Cl-exposed sample. Growth of the PCB-exposed sample is more pronounced than in the controls, whereas that of the 4-NP and TPT-Cl samples is markedly delayed. In addition, 4-NP and TPT-Cl samples show evident malformations. This can be appreciated in terms of both overall size and shape of the regenerate and differentiation of anatomical structures. *Bars* 240 μm (**e**, **g** and **h**); 250 μm (**f**)

4.3 Malformations

The morphological analysis under the stereomicroscope pointed out specific malformations in the exposed samples which could be attributed to individual compounds (Candia Carnevali et al. 2001a,b, 2003; Barbaglio et al. 2004). Results obtained from the different series of tests performed with PCBs, 4-NP and TPT-Cl, respectively, were in good agreement from a qualitative point of view. Appreciable malformations in terms of general morphology and external anatomy were observed in specimens exposed to 4-NP and TPT in comparison with controls (Fig. 4 c,d,g,h). In particular, during the advanced regenerative phase (1–2 weeks), the evident delayed development of the regenerate was expressed not only by the smaller overall size of the regenerating arm but also by a partial lack or abnormal development of its lateral pinnules (Fig. 4g,h). The same range of anomalies was observed with all the exposure concentrations tested. As far as TPT-Cl exposure experiments are concerned, except for the 50 ng/l samples, all the exposed samples showed evident anomalies in terms of both external malformations in their overall shape and anatomical features. Beside a generalized reduced growth of the regenerative blastema, remarkable atypical features could be seen in the regenerating arms of the exposed specimens which appeared to be twisted and coiled or unusually tuft-shaped (Fig. 4 c,h). This aberrant anatomy of the regenerate was consistent with the presence of relevant anomalies in the corresponding histological sections (see below), which were characterized by an unusual pattern of specific tissues, with particular reference to a pronounced abnormal development of the skeletal components.

4.4 Histological Pattern

The effects observed at the level of general morphology can be correlated with relevant atypical features in terms of microscopic anatomy of both the stump and the regenerating arm. These alterations are the most sensitive index of ecologically relevant, individual-level responses such as growth and development (Hinton 1997). With all the test compounds employed so far, the histological sections, in spite of a general good preservation of tissue integrity in all the exposed samples, showed clear signs of anomalies, which were detectable since the early regenerative phase and became more and more evident at the more advanced regenerative stages (1 and 2 weeks post-amputation). It is significant that these histological alterations always involved the same target structures, but showed rather characteristic aspects that could be considered specific effects of the exposure to the individual pollutant (Candia Carnevali et al. 2001a,b, 2003; Barbaglio et al. 2004). Apart from a number of other minor atypical features, the following anomalies appeared to be particularly relevant:

1. Development of an atypical blastema (pseudo-blastema), flattened and/or ectopic, often including foreign non-blastemal elements (myocytes and/or skeletal spicules) (in 4-NP- and TPT-exposed samples; Fig. 5a,b).
2. Unusual hypertrophy and swelling of the coelomic canals of both the stump and the regenerate (particularly in PCB- and 4-NP-exposed samples; Fig. 5d,e,f,i).
3. Pronounced abnormal development of the skeletal components in the regenerate (particularly in TPT-exposed samples; Fig. 5 c).
4. Enhanced and prolonged phenomena of cell migration (coelomocytes, amoebocytes, phagocytes and granulocytes) evident at the level of both coelomic canals and tissues (in all the exposed samples; Fig. 5b,i,k). In particular, this intense migratory activity tended to involve a large number of granulocytes: their diffuse presence in the tissues, even at the advanced regenerative stages, and the massive unusual occurrence of degranulation phenomena in the stump tissues, particularly close to the coelomic canals, were distinctive features of all the exposed samples. Under standard conditions, in fact, the granule cells are extensively but limitedly employed during the repair processes, their degranulation processes occurring specifically at the level of the amputation surface.
5. Massive recruitment of dedifferentiated or semi-dedifferentiated cells, particularly myocytes, that were frequently involved in migration phenomena, particularly in the coelomic canals close to the amputation area (in all the exposed samples, but mostly in 4-NP and TPT samples; Fig. 5b,i,k). The muscles of the stump contributed extensively to this cell recruitment/migration through phenomena of tissue rearrangement and dedifferentiation during which the muscle fibres were massively replaced by other elements (coelomocytes and phagocytes; Fig. 5g,h,i,j). At TEM (Fig. 6a) many individual myocytes appeared to progressively dedifferentiate to acquire the features of undifferentiated coelomocytes actively involved in cell division (see below), whereas other myocytes were obviously involved in apoptosis (Fig. 6b). These processes of muscular dedifferentiation and turnover were always accompanied by related phenomena of massive cell migration/proliferation in the adjacent coelomic canals (Fig. 6 c). This is strongly in contrast to what was observed under standard conditions, where regeneration is typically accomplished by the contribution of undifferentiated stem cells which are responsible for the blastema formation.
6. Enhanced or inhibited cell proliferation. The first effect was particularly evident in PCB-exposed samples, the second in 4-NP- and in TPT-exposed samples. As indicated by our BrdU incorporation experiments, although cell proliferation in both exposed and control samples was always localized in the usual specific cytopoietic sites, mainly at the level of the apical blastema and the coelomic epithelium of both the regenerate and the stump, there was a substantial difference in labelling intensity and distribution among the different samples: the labelling in fact was much stronger and more widely distributed in PCB samples (Fig. 6f,h), whereas it was

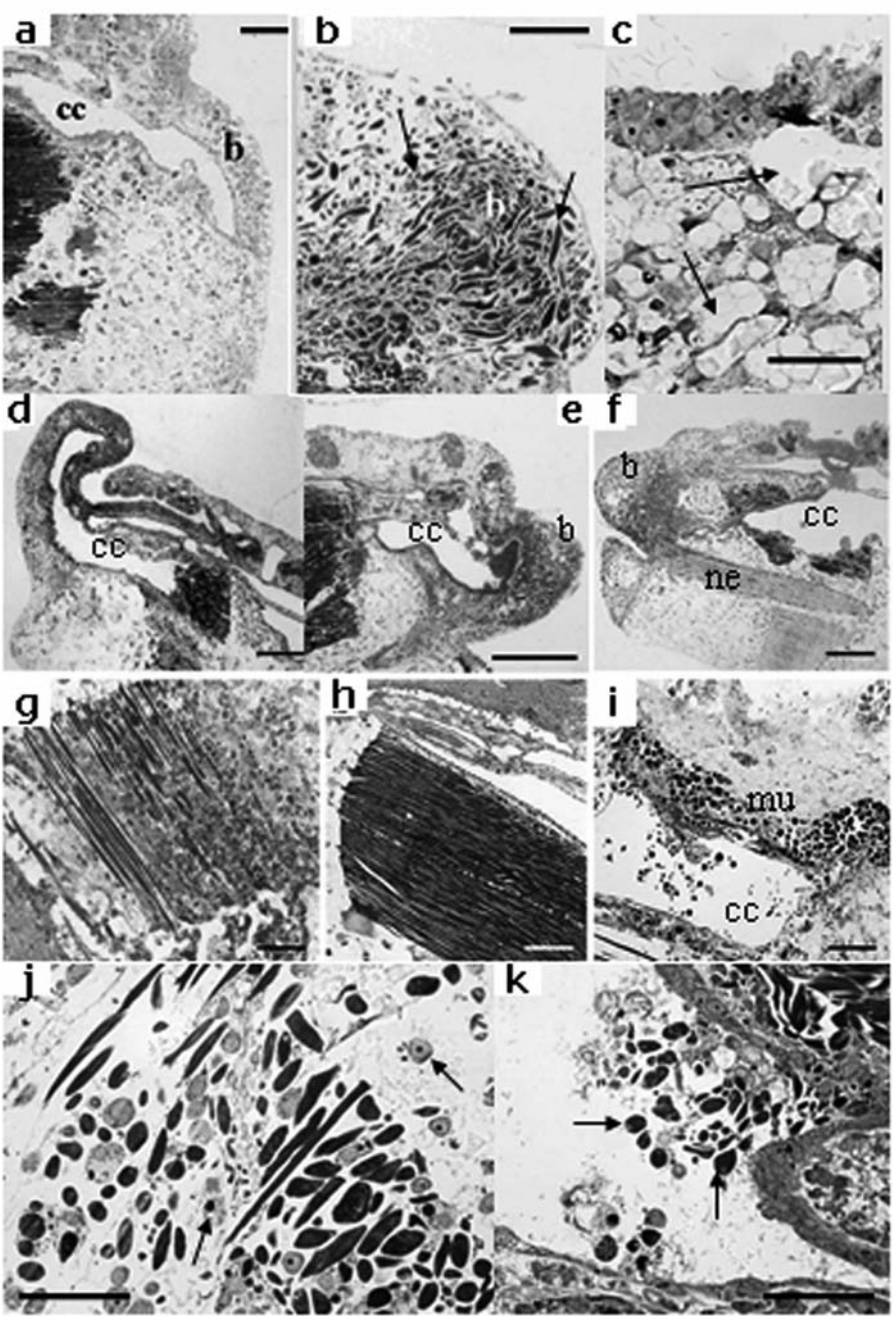


Fig. 5a–k. LM sagittal sections of control and exposed regenerating arms of *Antedon mediterranea* at different stages. Exposure concentrations: PCB: 14 ng/l; 4-NP: 2 µg/l; TPT-Cl: 100 or 225 ng/l. **a–c** Details of the blastema regions at 72 h pa (post-amputation): control (**a**) and TPT-Cl samples (**b**, **c**). In **b**, an atypical pseudo-blastema which mainly consists of ectopic migratory myocytes (*arrows*) is evident; **c** shows the unusual presence of well-

rather modest and less diffuse in 4-NP and TPT samples (Fig. 6g) with respect to the controls. Unexpectedly, in PCB-exposed specimens, the labelling involved to a relevant extent also the muscles of the stump (Fig. 6h). This seems to indicate that, at least in the PCB-exposed samples, the muscles can directly provide a significant contribution to regeneration also in terms of cell proliferation.

7. Recycling and turnover of the stump tissues, namely the endoskeleton and the connective tissue (mostly in PCB- and 4-NP-exposed samples; Fig. 6d). These phenomena were significantly different from those related to the muscles and involved extensive degenerative phenomena which led to vacuolization/vesiculation of both the extracellular matrix and the cells (fibroblasts or scleroblasts respectively). The massive presence of phagocytes at advanced regenerative stages of 2 weeks indicates that these tissues are employed as a secondary indirect source of reserve materials for new synthesis.
8. Atypical ultrastructural features of specific cell types, including the blastemal cells (mostly in PCB- and 4-NP-exposed samples; Fig. 6e). In particular, a marked development of endoplasmic reticulum (both RER and SER) and Golgi complexes and an unusual abundance of lipid granules and empty vacuoles were typical features of the exposed samples and could be correlated with a cytological pattern of steroid dysfunction. Interestingly, in the exposed samples, besides these histological and cytological features, the integrity/preservation of other tissues of both the stump and regenerate (epithelia, nerve tissue, etc.) was rather good, even in the long-tem exposed samples of 2 weeks post-amputation, without any significant morphological variation with respect to the standard conditions seen in normal regeneration;
9. Possible induction of protective biochemical responses. Immunocytochemical results showed, in fact, that in PCB- and 4-NP-exposed samples there was an appreciable increase in the expression pattern of specific

developed skeletal spicules in the blastema (*arrows*). **a** and **b**, *bar* 100 μm; **c** 20 μm. **d–f** Exposed regenerating samples of 1 week pa: **d** PCB; **e** TPT-Cl; **f** 4-NP. Abnormal growth of the regenerate is evident in PCB samples. In contrast, the regenerative blastema is heterogeneous and poorly developed in TPT-Cl and 4-NP specimens. In all the exposed samples the coelomic canals (*cc*) are hypertrophic. *b* Blastema; *ne* nerve. *Bars* 200 μm. **g–i** Exposed and control samples at 1 week pa: **g** PCB; **h** control; **i** 4-NP. Details of the muscle bundles of the stump. In contrast to the compact structure of the control muscle, the PCB- and 4-NP-exposed samples show extensive muscle rearrangement/dedifferentiation. *cc* Coelomic canal; *mu* muscle. *Bars* 30 μm. **j**, **k** Details of 4-NP-exposed samples of 1 week pa. **j** The muscle bundle consists of semi-dedifferentiated myocytes and undifferentiated coelomocytes (*arrows*); **k** the coelomic canals of the stump are involved in extensive cell proliferation/ migration (*arrows*). A number of myocytes are released through the coelomic wall into the lumen. *Bars* 20 μm

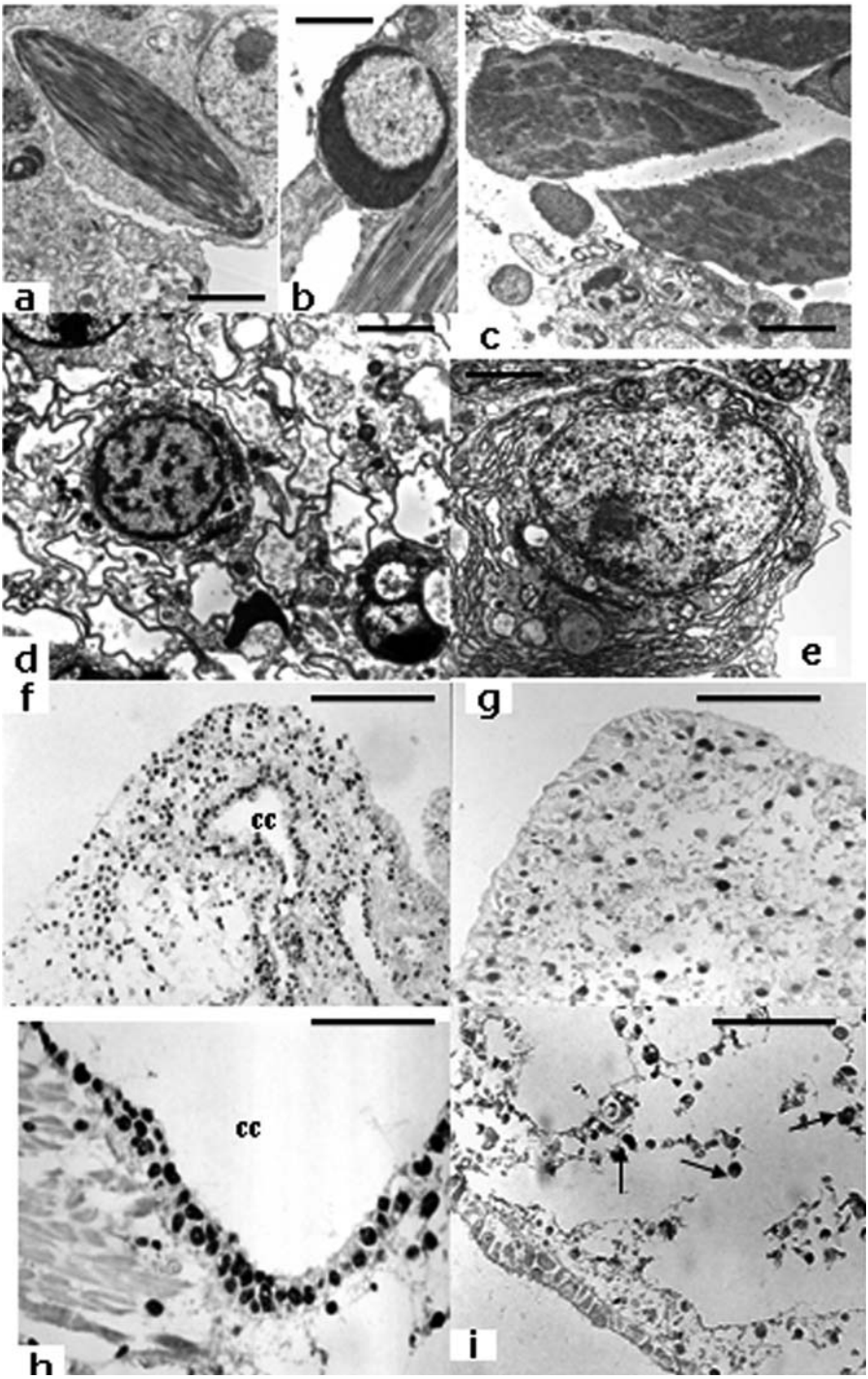

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Fig. 6. TEM and ICC sections of control and exposed regenerating arms of *Antedon mediterranea* at different stages. Exposure concentrations: PCB: 14 ng/l; 4-NP: 2 µg/l; TPT-Cl: 100 or 225 ng/l. **a–d** TEM details of 1 and 2 weeks exposed samples. *Bars* 2 µm. PCB (**a**, **e**, **f**), 4-NP (**b**, **c**). **a** Detail of the muscle bundle of the stump. **b** Myocyte showing a typical apoptotic nucleus. **c** Migratory semi-dedifferentiated myocytes inside the coelomic canal. **d** Connective tissue of the stump showing extensive processes of cellular rearrangement/vacuolization. **e** Blastemal cell showing unusual development of endoplasmic reticulum and Golgi complexes. *Bar* 1 µm. **f–h** ICC for BrdU. The number of BrdU-labelled cells is massive in the blastema of PCB samples (**f**) and rather limited in that of 4-NP samples (**g**). A strong and extensive labelling is also found in the coelomic epithelium (*cc*) of PCB samples (**h**). **i** ICC for cytochrome P450. The details show an intense immunoreaction in many cells (*arrows*) at the level of the tissues of the stump, particularly in connective and skeletal tissue. *Bars*: **f** 25 µm; **g** 30 µm; **h** 10 µm; **i** 30 µm

enzymes such as the microsomal cytochrome P-450 mono-oxygenase system, i.e. the main enzymes responsible for biotransformation and metabolism of the majority of lipophilic xenobiotics (den Besten 1998). Interestingly, the reaction was intense and diffuse in the tissues of the stump more involved in turnover activity (Fig. 6i).

5 Conclusions and Future Prospects

In the light of the present results, the regenerative processes of crinoids appear to provide valuable and flexible experimental models for studies exploring the effects of exposure to exogenous substances on the regenerative growth with particular reference to endocrine disrupter contaminants (EDs), which induce significant dysfunctions in terms of processes of growth and development from the whole organism level up to the tissue, cellular and molecular level.

In particular the experiments of prolonged exposure to typical ED compounds, such as PCBs, 4-NP and TPT, employed at low concentrations reproducing the contamination levels of moderately polluted coastal zones (Geyer et al. 1994), indicate significant variations in timing and intrinsic mechanisms of regeneration in terms of:

- accelerated or delayed growth
- enhanced or inhibited cell proliferation
- massive and prolonged cell migration
- extensive cell/tissue recycling and rearrangement
- aberrant histogenesis
- cytological disorder
- induction of protective biochemical responses

These effects are associated to a rapid and persistent bioaccumulation of all the test compounds in the animal tissues (Candia Carnevali et al., 2001a,b; Dagnac et al. unpublished; Tremolada et al. in press). Although these aspects often indicate specific and contrasting effects caused by the individual contaminant (Barbaglio et al. 2004; Candia Carnevali et al. 2001a,b; 2003), they point out that all these compounds 1) affect growth and development by interfering with the same basic cellular mechanisms of regeneration, such as cell proliferation, migration and differentiation/dedifferentiation, which are possibly controlled and stimulated, directly or indirectly, by steroid hormones (Marsh and Walker 1995); 2) can induce a number of significant modificantions in timing, modalities and pattern of arm regeneration which, in terms of histopathology, can be interpreted in the light of a significant activation of cell mechanisms related to steroid synthesis/metabolism and/or to specific detoxification processes (den Besten 1998; Schoenmakers 1980). The possible effects of exposure tests with suitable mixture of ED compounds, such as PCBs, 4-NP, organotins and others, could be an intriguing point to investigate in a further step of the research work in order to progress in exploring in realistic terms the potential ecological significance of endocrine disruption in marine invertebrates.

Taking into account the increasing importance for our future prospects of life to determine the impacts of chemicals in the environment, the bulk of our results not only presents a new valuable model for specific ecotoxicological applications, but also clearly reveal how appropriate can be to employ an integrated approach centered on individual responses at more than one level of biological organization, from cell to whole animals, for testing the effects of exposure to insidious exogenous substances on growth and development. Useless to say, our multilevel approach, can be obviously helpful to identify, assess and validate suitable biomarkers at tissue and cellular level. In addition this approach can also be naturally expanded in the area of *in vitro* aquatic toxicology if suitably associated to applications of in vitro techniques, a field which needs urgently to be developed in aquatic invertebrates for conducting controlled laboratory experiments on well-characterized cell lines also derived from adult regenerating animals. The potential for *in vitro* techniques in this area is clearly shown by Matranga et al., this book. Last but not least, our approach can also help to throw light on basic problems such as that of the regeneration-competent cells, in terms of origin (stem cells or dedifferentiated cells), activities (proliferation and/or migration) and fate (derived cell lineages), or that of the functional implication of regulatory mitogenic or morphogenic factors. An integrated understanding of the cellular and molecular basis of these processes emerging from basic research associated to complementary experimental approaches, such as a modern ecotoxicological approach, in vivo and in vitro, appears in fact to be quite timely and appropriate. The crucial point of the "progenitor" elements involved in regenerative processes, in terms of cell recruitment, sources and fate, and totipotentiality,

pluripotentiality or unipotentiality, is a central problem, particularly relevant for its topical interest, which deserves to be explored appropriately also in experimental models less commonly used but potentially very amenable for this approach. The identification of the cellular events induced by wounding and repair in regenerating animals in normal and extreme conditions of environmental stress can significantly help to understand the conditions and factors that allow developmental fields to be established de novo in terminally differentiated tissues. The stimulation of regeneration of new tissues from old tissues in vivo, constitutes, in fact, an emerging and exciting interdisciplinary field called "regenerative biology" (Stocum 1998) whose wide potential of expansion is strictly depending on the knowledge of the fundamental mechanisms at the cellular and molecular level. The identification of the distribution and characteristics of the reserve of progenitor cells and of the signals, stimulatory or inhibitory, to regeneration will be major avenues of research in the new field of regenerative medicine (Pearson 2001) and, in our attempt to regenerate human tissues in vivo, we can learn from crinoids how to induce the formation of progenitor cells by cell dedifferentiation.

On the whole our ecotoxicological approach throw new light into the wide field of environmental regulation of development by confirming that also regenerative development is "critically keyed to the environment" (Gilbert 1997). The bulk of results obtained so far provide significant indications on sub-lethal effects of exposure to ED compounds and mechanisms of toxicity related to developmental physiology, in terms of both regulatory mechanisms of growth, morphogenesis, differentiation and morphological anomalies at whole organism, tissue and cellular level. In conclusion, the regenerative response of crinoids can be considered a new, quite original and sensitive test for studying ecotoxicological effects of persistent ED pollutants and assessing their disrupting activity, which not only perfectly responds to the 5-*R* requisites indicated by Hopkin (1993): *R*elevant, *R*obust, *R*eliable, *R*esponsive, *R*eproducible, but even extends the definition of the ideal ecotoxicological model by adding a possible sixth *R* : *R*egenerating.

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