

## REVIEW ARTICLE

**Gnotobiotically grown aquatic animals: opportunities to investigate host–microbe interactions**A. Marques<sup>1</sup>, F. Ollevier<sup>2</sup>, W. Verstraete<sup>3</sup>, P. Sorgeloos<sup>1</sup> and P. Bossier<sup>1</sup><sup>1</sup> Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium<sup>2</sup> Laboratory of Aquatic Ecology, Faculty of Science, Catholic University of Leuven, Leuven, Belgium<sup>3</sup> Laboratory of Microbial Ecology and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium**Keywords**

aquatic animals, axenic, gnotobiotic, host–microbe interactions.

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**Abstract**

The culture of aquatic organisms is still hampered by the occurrence of unpredictable diseases in their early life stages, which are responsible for massive mortalities and considerable economic losses. A better understanding of the host–microbe interactions is certainly essential to develop effective solutions of disease control for the aquaculture industry. As demonstrated in terrestrial animals, the use of gnotobiotic systems (animals cultured in axenic conditions or with a known microflora) can be an excellent tool to extend the understanding of the mechanisms involved in host–microbe interactions and to evaluate new treatments of disease control. Several aquatic animals were cultured so far in germ-free conditions, such as fish, molluscs, crustaceans, rotifers, echinoderms, cnidarians, turbellarians, ascidians and echiurans. The aim of the present review is to recapitulate the findings obtained with gnotobiotic aquatic animals over the last decades, with special emphasis to the host–microbe interactions, as well as the perspectives for future research in this field. In addition, the procedures utilized to culture axenic aquatic animals and to verify contaminations are summarized, and the standardization of these procedures is proposed.

**Introduction**

Aquatic animals are nutritionally very important for human consumption, as they are excellent sources of proteins, trace elements and polyunsaturated fatty acids (Ruxton *et al.* 2005). Consequently, there is a global increase in the demand of fish and shellfish products (FAO 2004). As fisheries cannot provide sufficient amounts of aquatic products to fulfil the consumer needs, aquaculture is the crucial alternative resource.

Aquaculture has become more important and intensive over the last decades and is presently the fastest growing food production industry with an average yearly growth rate of more than 6% over the last 20 years (Brugère and Ridler 2004). Despite the technological improvements that allowed the expansion of aquaculture over the years, diseases are still a major constraint (FAO 2004). Unpredictable massive mortalities still occur in early life stages of

aquatic organisms (especially for most marine fish and shellfish species) as a result of the proliferation of pathogenic and opportunistic micro-organisms in the culture systems, being responsible for considerable economic losses. Nowadays, several environment-friendly prophylactic and preventive methods are being developed to control such diseases and to maintain a healthy microbial environment in aquaculture systems (e.g. probiotics, immunostimulants, antimicrobial peptides, quorum sensing analysis) (Vadstein 1997; Verschuere *et al.* 2000a; Bachère 2003; Defoirdt *et al.* 2005). However, applications of these technologies must be based on thorough understanding of mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies looking in detail at the host–microbial interactions. A key experimental strategy to study these interactions is to first define the animal functioning in the absence of all micro-organisms (i.e. under germ-free or

gnotobiotic conditions) and then to evaluate the effects of adding a single or defined population of microbes or certain compounds (Gordon and Pesti 1971).

Studies performed with gnotobiotic aquatic organisms are still scarce and mostly disperse. In addition, some constraints still hamper the wide use of gnotobiotic aquatic animals for research purposes, such as: the diversity of disinfection/sterilization methods to produce axenic organisms, and the difficulty to assure the complete axenicity of a culture system. Several aquatic animals were cultured in germ-free (or near-axenic) conditions, such as fish, molluscs, crustaceans, rotifers, echinoderms, cnidarians, turbellarians, ascidians and echiurans. Findings obtained with gnotobiotic aquatic animals can be mainly separated in three categories: (i) nutritional requirements; (ii) host–microbe interactions; (iii) or metabolic functions.

The aim of the present review is to provide a critical overview of the findings obtained with gnotobiotic aquatic animals over the last decades. Particular attention will be paid to the host–microbe interactions, as well as to the methods used to obtain axenic organisms and to verify contaminations. Finally, some guidelines are also presented for future research in this important field.

### Advantages and constraints of gnotobiology

Although metabolic activities of micro-organisms influence the animal behaviour, a primary prerequisite for studying diseases is the absence of earlier exposition to the causal microbe or the absence of any antibody derived from preceding exposition (Dufty 1976). Therefore, the composition of the associated microbiota of laboratory animals should not be left to chance. In addition, when using non-axenic experimental setups the complex interaction between the microbial community (MC) and its host is often difficult to disentangle. The use of gnotobiotic organisms allows an increased control of variables, enhanced reproducibility of results, and more accurate experimental designs by separating an animal into host and microbiota (Pleasant 1973).

The very uniqueness of a gnotobiotic organism confers its own limitations. The major constraints of such artificial culture conditions are: (i) the technical complexity and high cost required to perform such cultures, especially in ‘higher’ organisms that require more diverse and complex equipment, foods and environmental conditions; (ii) the specialized skills required to handle and maintain such organisms free of contaminants; (iii) the difficulty to reproduce under laboratory conditions the complex and variable aspects of ‘natural’ environments (only certain aspects can be analysed); and (iv) the intrinsic characteristics of germ-free organisms. In germ-free creatures, the

cellular and humeral defence system is usually underdeveloped compared with conventional (non-gnotobiotic) animals (Dufty 1976), meaning that their response to treatments may be atypical to events normally occurring under field conditions. These differences are likely due to the microbiota present in conventional animals, which affects the nutritional requirements, physiology, metabolism and anatomy of the host, as well as suppress the establishment of some pathogens and exacerbate the effect of others (Gordon and Pesti 1971). By acting on substances in the alimentary canal, bacteria can also release toxic materials and alter drugs (Goldman 1978).

Nevertheless, many differences between gnotobiotic and conventional animals (e.g. growth and metabolism) were already determined, especially in terrestrial animals, as a result of improvements in rearing methods and development of better diets (Trexler 1978). To reduce further differences, germ-free organisms can be colonized with functionally known symbiotic microflora obtained from conventional organisms. Therefore, it is essential to determine which microbes are fundamental for the development of each target organism.

### Procedures to obtain and maintain gnotobiotic aquatic animals

A better understanding of several biological functions in aquatic organisms (e.g. host–microbe interactions) can only be achieved with animals cultured in fully controlled conditions. Nowadays, there is a huge diversity of disinfection/sterilization methods to produce axenic aquatic organisms. The appropriate disinfection methods depend mostly on the animal group studied and on their developmental stage (e.g. egg, tissue, zygote, gametes, juvenile and adults) (see Tables 1 and 2). Taking into account the results obtained in previous studies, the present synopsis proposes the standardization of the disinfection procedures according to the developmental stage of the animal to be axenised.

So far, ‘bacteria-free’ aquatic animals were obtained mainly by three different ways: (i) removing eggs, newborn organisms, larvae or embryos not yet contaminated with microbes from pregnant females by surgery (e.g. Manahan *et al.* 1983); (ii) using eggs, newborn organisms, larvae or embryos with reduced levels of contamination that are treated with antibiotics (e.g. Munro *et al.* 1995) or, when possible, using strong antibacterial solutions to eliminate microbes (e.g. Douillet and Holt 1994); or (iii) using conventional eggs, newborn organisms, larvae or embryos that initially undergo a series of washings to dilute the unwanted accompanying microbes followed (or not) by antibiotic or other chemical treatments to completely eliminate microbes (e.g. Rahat and Diementman

**Table 1** Overview of studies performed with germ-free freshwater fish, marine fish and Molluscs, ordered chronologically by species

Species	Methods used to obtain axenic organisms			Exposure	Methods to verify contamination		Nature of study	References
	Target	Method	Amount		Methods to verify contamination			
<b>Freshwater fishes</b>								
<i>Salmo irideus</i>	Eggs	Acridiflavine or sulfo-methiolate	0.015% 0.185%	30 min	FIB (RT)	Effect of germicides on bacteria and eggs	Gee and Sarles (1942)	
<i>Salmo fario</i>								
<i>Salvelinus fontinalis</i>	Adults	Povidone-iodine	1% iodine	Bath	BHIB, TSB, GFB (13 and 30°C)	Axenic culture	Trust (1974)	
<i>Oncorhynchus kisutch</i>	Eggs	Sterile tools						
<i>O. keta</i>								
<i>O. nerka</i>								
<i>Salmo aquabonito</i>								
<i>Salmo gairdneri</i>								
<i>Salvelinus fontinalis</i>								
<i>Danio rerio</i>	Adults	Betadine	10%	2 min	NB, BHIB, SDB	Axenic culture	Rawls et al. (2004)	
	Eggs	Ampicillin + Kan + amphotericin B	100 µg + 5 µg + 250 ng (ml <sup>-1</sup> )	6 h	(28°C and 37°C)	Morphology		
	Eggs	Betadine	0.1%	2 min		Bacteria vs fish		
	Eggs	NaOCl	0.003%	20 min				
<b>Marine fishes</b>								
<i>Gadus morhua</i>	Eggs	Sterile tools	nr	nr	nr	Microbes vs eggs	Hansen and Olafsen (1989)	
<i>Hippoglossus hippoglossus</i>	Eggs	Hydrogen peroxide	3%	5 min	ZB, ZA, TSPSB, TSPSA (nr°C), DAPI	Effect of germicides to obtain bacteria-free fish	Douillet and Holt (1994)	
<i>Sciaenops ocellatus</i>								
<i>Ocyurus chrysurus</i>								
<i>Cynoscion nebulosus</i>								
<i>Gadus morhua</i>	Eggs	Glutaraldehyde or NaOCl (5% active chlorine)	25% (800 mg) 100 mg (ml <sup>-1</sup> )	2.5–10 min	M-65 SWA (10°C)	Effect of germicides on bacteria and eggs	Salvesen and Vadstein (1995)	
<i>Hippoglossus hippoglossus</i>								
<i>Pleuronectes platessa</i>	Eggs	OA + Kan + Ery + Pen G + Stre	10 + 10 + 10 + 150 + 75 (mg ml <sup>-1</sup> )	24 h	BHIB (1% NaCl) (20°C)	Microbes vs larvae	Munro et al. (1995)	
<i>Scophthalmus maximus</i>	Eggs	Glutaraldehyde	25%	10 min	MA, MB (6°C), DAPI	Microbes vs larvae	Verner-Jeffreys et al. (2003)	
<i>Hippoglossus hippoglossus</i>	Eggs	OA + Kan + Ery + Pen G + Stre	10 + 10 + 10 + 150 + 75 (mg ml <sup>-1</sup> )	Overnight				
	Eggs	Peracetic Acid	0.02%	20 s				
<b>Molluscs</b>								
<i>Mercenaria mercenaria</i>	Eggs	Pen G + Stre S + Chl	100 + 100 + 50 (mg l <sup>-1</sup> )	24–36 h	SWNB (nr°C)	Microbes vs larvae	Guillard (1959)	
	Eggs	Sterile seawater + ST	-	Bath	nr	?	Hidu and Tubiash (1963)	
<i>Crassostrea gigas</i>	Adults	NaOCl	1%	Bath	MB (25°C), DAPI	Microbes vs larvae	Douillet and Langdon (1993)	
	Adults	Betadine	nr	5 min	Droop E6 (25°C), AO	Microbes vs larvae	Le Deuff et al. (1994, 1996; Renault et al. (1995)	
	Eggs	OA + Kan + Ery + Stre S + Flu + Pen	0.1 + 0.1 + 0.1 + 0.1(mg l <sup>-1</sup> ) + 30 µg l <sup>-1</sup> + 100 units l <sup>-1</sup>	3 days				
<i>Haliotis midae</i>	Juveniles	Amp + Chl + Cef	600 + 250 + 250 (µg ml <sup>-1</sup> )	72 h	MA (22°C), DAPI, SEM	Microbes vs larvae	Erasmus et al. (1997)	

**Table 1** Continued

Species	Methods used to obtain axenic organisms			Methods to verify contamination		References
	Target	Method	Amount	Exposure	Nature of study	
<i>C. gigas</i>	Adults	Betadine	nr	5 min	Droop E6 (25°C), AO	Arzul <i>et al.</i> (2001)
<i>C. angulata</i>	Eggs	OA + Kan + Ery + Stre S + Flu + Pen	0.1 + 0.1 + 0.1 + 0.1 (mg l <sup>-1</sup> ) + 30 µg l <sup>-1</sup> + 100 units l <sup>-1</sup>	3 days		

?, not found; Al, alcohol; Amp, ampicillin; AO, acridine orange staining microscopy; BHLB, brain-heart infusion broth; Cef, cefotaxime; Chl, chloramphenicol; DAPI, 4',6-diamidino-2-phenylindole staining microscopy; Ery, erythromycin; FIB, fish infusion broth; Flu, flumequine; GFB, glucose fermentation broth; Kan, kanamycin; MA, marine broth; NaCl, sodium chloride; NaOCl, sodium hypochlorite; NB, nutrient broth; nr, no reference; OA, oxolinic acid; Pen, penicillin; Pen G, penicillin G; RT, room temperature; SDB, Sabouraud dextrose broth; SEM, scanning electron microscopy; Stre, streptomycin; Stre S, streptomycin sulphate; SWA, seawater agar; SWNB, seawater nutrient broth; TSB, trypticase soy broth; TSPSA, tryptic soy plus salt agar; TSPSB, tryptic soy plus salt broth; ZA, Zobell agar; ZB, Zobell broth.

1982). The first method used to obtain axenic zygotes of aquatic animals by killing the parents is commonly used in freshwater fish, some molluscs, echinoderms, cnidarians and echinurans. This method allows obtaining eggs or larvae with limited amounts of contaminations, as the gonads are usually axenic or present reduced levels of contaminants. However, this technique presents some constraints, as it requires killing the adult organisms. To avoid such practice, gametes, eggs and larvae could be disinfected (as performed in the second method with marine fish, molluscs, crustaceans, rotifers and turbellarians).

Some organisms, like *Artemia*, switch to a particular form of reproduction (oviparous reproduction) when the environmental conditions are adverse, i.e. developing embryos enclosed in multi-layer shells (also called cysts) that protect them against exterior aggressions. This particular characteristic allows the use of strong oxidizing agents at high concentrations (e.g. sodium hypochlorite) to disinfect the cysts very efficiently and with a very high reproducibility (Marques *et al.* 2004a,b). However, as most organisms do not possess an efficient shell able to protect embryos against environmental aggressions, they can only be effectively disinfected with less aggressive antibacterial solutions at low concentrations (such as merthiolate, glutaraldehyde, sodium hypochlorite, hydrogen peroxide and ethanol) and/or antibiotics. In most cases these disinfection procedures present lower reproducibility in comparison with the use of higher amounts of strong oxidizing agents. Zygotes or larval stages collected without killing the parents certainly carry more contaminants, which are more difficult to eliminate than early life stages collected directly from killed parents. The concentration of contaminants can be significantly reduced if these larval stages are washed (using the 'dilution method') before using an antibacterial solution or antibiotic to complete the disinfection procedure (third method).

Although several authors (e.g. Murphy 1970) advocated the use of the 'dilution method' as a single technique to obtain axenic aquatic animals, its applicability for routine production of axenic animals is certainly hampered by its irreproducibility, as its efficiency is dependent on the amounts of contaminants present in the culture medium.

Whenever antibiotics or antibacterial solutions are required to disinfect aquatic organisms, special attention should be taken to assure that they are completely removed before starting any experiment. In addition, tests should be performed to assure that antibiotics or antibacterial solutions do not affect the target organism in any circumstance. If the animals present weaker health status (and consequently poorer development) after the disinfection

**Table 2** Overview of studies performed with germ-free crustaceans, rotifers, echinoderms and cnidarians, ordered chronologically by species

Species	Methods used to obtain axenic organisms				Exposure	Amounts	Methods to verify contamination		Study purpose	References
	Target	Methods	Methods	Amounts			Methods to verify contamination	Amounts		
<b>Crustaceans</b>										
<i>Artemia salina</i>	Cysts	Merthiolate + aerosol OT		1 ppt + 10% 2 ml l <sup>-1</sup>	10 min	STP medium (22–26°C)		Nutritional requirements; metabolic functions	Provasoli and D'Agostino (1969)	
<i>Daphnia magna</i>	Adults	RW + sterile tools		–	Baths	TG (nr°C), M		Nutritional requirements	Murphy (1970)	
<i>Moina macrocopa</i>										
<i>Artemia</i> sp.	Cysts	Merthiolate		1ppt	10 min	2% YEG (RT); STM (nr°C); DBM (nr°C); MA (nr°C)		Nutritional requirements <i>Vibrio</i> vs <i>Artemia</i>	Gunther and Catena (1980), Douillet (1987)	
<i>Artemia salina</i>	Cysts	NaOCl (2% chlorine)		20%	15 min	4% PP (33°C)		Feed to axenic hydra	Rahat and Diementman (1982)	
<i>Artemia franciscana</i>	Cysts	NaOCl + merthiolate		nr + 0.05%	nr + bath	nr		Microbes vs nauplii	Rico-Mora and Voltolina (1995)	
	Cysts	Decapsulation		nr	nr	MA, TCBS (30°C)		Microbes vs nauplii	Gomez-Gil et al. (1998)	
	Cysts	Chl + Tri + Sfm		30 + 40 + 8 (mg l <sup>-1</sup> )	24 h			Microbes vs nauplii	Verschuere et al. (1999, 2000b)	
	Cysts	Merthiolate		1 g l <sup>-1</sup>	10 min	MB, MA (28°C)		Microbes vs nauplii	Jung and Hagiwara (2001)	
<i>T. japonicus</i>	Adults/Larvae	Dhstre + Pen G + Pol B S; Tet + Chl + Neo		5000 + 5000 units + 150 units + 250 + 50 + 250 (µg ml <sup>-1</sup> )	2 baths (30 min)	STP medium or MA (RT)		Nutritional requirements Microbe vs. larvae		
<i>Artemia franciscana</i>	Cysts	Cloralex (active chlorine)		60 g l <sup>-1</sup>	10 min	MA, M (30°C)		Microbes vs nauplii	Orozco-Medina et al. (2002)	
	Cysts	Decapsulation		nr	nr	TSA + 2.0% NaCl (30°C), DTAF		Microbes vs nauplii	Soto-Rodriguez et al. (2003)	
	Cysts	Chl + Tri + Sfm		30 + 40 + 8 (mg l <sup>-1</sup> )	24 h			Microbes vs nauplii, feed to axenic halibut	Verner-Jeffreys et al. (2003)	
	Cysts	NaOCl + NaOH + HCl		10% + 0.25 mol l <sup>-1</sup> + 0.1 mol l <sup>-1</sup>	nr	MA, MB (6°C), DAPI		Nutritional requirements; microbes vs nauplii	Marques et al. (2004a,b, 2005, 2006), Defoirdt et al. (2005)	
	Cysts	NaOCl + NaOH + Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>		50 ml (32%) + 3 ml (50%) + 90 ml (tap water) + 75 ml (10 g l <sup>-1</sup> )	150 s Bath	MA (28°C) and MTT (RT); or MB (28°C)		Microbes vs rotifers	Meadow and Barrows (1971)	
<b>Rotifers</b>								Microbes vs rotifers	Hagiwara et al. (1994)	
<i>Philodina acuticornis</i>	R. eggs	Kan S + Col Na + Cep Na		1 + 1 + 1 (mg ml <sup>-1</sup> )	5–6 h	TSA, DA (nr°C)		Microbes vs rotifers		
<i>Brachionus plicatilis</i>	R. eggs	Dhstre + Pen G + Pol B S + Tet + Chl + Neo		5 + 5000 u + 0.15 u + 0.25 + 0.05 + 0.25 (mg ml <sup>-1</sup> )/0.5 + 0.25 ppm	2 h + Bath or 60 + 30 min	STP (nr°C)		Microbes vs rotifers		
	R. eggs	NaOCl		10 + 10 + 10 + 150 + 75 (mg ml <sup>-1</sup> )	24 h	BHIB (1% NaCl) (20°C)		Microbes vs rotifers, feed to axenic turbot	Munro et al. (1995)	
<i>B. plicatilis</i>	R. eggs	OA + Kan + Ery + Pen G + Stre		5, 1, 0.5, 0.1 or 0.05%	1, 3 or 5 min	MA (25°C), DAPI		Microbes vs rotifers	Douillet (1998, 2000a,b)	
<i>B. rotundiformis</i>	R. eggs	NaOCl						Microbes vs rotifers		
<i>B. plicatilis</i>	R. eggs	Merthiolate or glutaraldehyde		100 mg l <sup>-1</sup> or 0.05 µl l <sup>-1</sup>	10 min or 6 h	MA (28°C)		Rotifers vs Copepods vs. microbes	Rombaut et al. (1999)	
<i>B. rotundiformis</i>	R. eggs	Dhstre + Pen G + Pol B S + Tet + Chl + Neo		5 + 5000 U + 0.15 U + 0.25 + 0.05 + 0.25 (mg ml <sup>-1</sup> )	2 h + Bath	MA (nr°C)			Jung and Hagiwara (2001)	

**Table 2** Continued

Species	Methods used to obtain axenic organisms			Amounts	Exposure	Methods to verify contamination	Study purpose	References
	Target	Methods	Exposure					
<i>B. plicatilis</i>	R. eggs	Tri + Sx	24 h	100–500 µl (40 + 8 mg ml <sup>-1</sup> )	BHIB (30°C)	Effect of germicides on bacteria and eggs	Martínez-Díaz <i>et al.</i> (2003)	
<b>Echinoderms</b>								
<i>Strongylocentrotus purpuratus</i>	R. eggs	Glutaraldehyde	1–2 h	50–100 ppm	MA (28°C) and MTT (RT)	Axenic culture, Microbes vs rotifers	Tinh <i>et al.</i> (2006)	
<b>Cnidarians</b>								
<i>Hydra viridis</i>	Adults	Ethanol	Bath	95%	ZB (n°C), AO	Nutritional requirements; Metabolic functions	Manahan <i>et al.</i> (1983)	
<i>H. vulgaris</i>	Adults	RW + Pen + Stre + Neo + Chl or Rif	48 h	100 µg ml <sup>-1</sup> each	PP (33°C)	Microbes vs host	Rahat and Diementman (1982)	

AO, acridine orange staining microscopy; BHIB, brain-heart infusion broth; Cep Na, sodium cephalothin; Chl, chloramphenicol; Col Na, sodium colistimethate; DA, desoxycholate agar; DAPI, 4',6-diamidino-2-phenylindole staining microscopy; DBM, Difco broth mixture; DTAF, 5-(4,6-dichlorotriazin-2-yl) aminofluorescein staining microscopy; Ery, erythromycin; HCl, chloridric acid; Kan, kanamycin; Kan S, kanamycin sulphate; M, microscopy; MA, marine agar; MB, marine broth; MITT, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide staining; Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, sodium thiosulphate; NaCl, sodium chloride; NaOH, sodium hydroxide; Neo, neomycin; nr, no reference; OA, oxolinic acid; Pen, penicillin; Pen G, penicillin G; Pol B S, polymyxin B sulphate; PP, proteose peptone medium; Rif, rifampicin; RT, room temperature; RW, repeated washings; Sfm, sulfamethoxazole; STM, sterility test medium; STP, seawater with trypticase, yeast extract and sugar; Stre, streptomycin; Sx, sulfametoxalose; TCBS, thiosulphate citrate bile sucrose agar; Tet, tetracycline; TG, thioglycolate broth; Tri, trimethoprim; TSA, trypticase soy agar; YEG, yeast extract glucose solution; ZB, Zobell broth.

tion procedure, their use in future experiments is certainly compromised as their behaviour can significantly diverge from non-axenic organisms of the same specie. To our knowledge, few studies were performed so far on the effect of germicides to gnotobiotic aquatic organisms, mainly with freshwater (e.g. Gee and Sarles 1942) and marine fish (e.g. Salvesen and Vadstein 1995).

In most studies performed so far with axenic aquatic animals, the reproducibility of the disinfection procedure is either not determined or limited, while only few studies reported high reproducibility of the disinfection procedure (e.g. Trust 1974; Douillet 1998, 2000a,b; Marques *et al.* 2004a,b). A scanning of the literature showed that the media used to culture gnotobiotic aquatic organisms are generally autoclaved to eliminate contaminants from the culture medium. Autoclaving effectively kills bacteria, but does not remove them from the media, thus the gnotobiotic organisms can use dead bacteria as feed, eventually affecting the experiments' reproducibility. According to findings obtained by Marques *et al.* (2005), dead bacteria even provided in small amounts can considerably change the performance of gnotobiotic *Artemia*. Therefore, the culture media of gnotobiotic aquatic organisms and of their live feed needs to be filtered (0.22 µm) before autoclaving. Only in this way, dead bacteria can be effectively removed from the media and the experiments' reproducibility improved.

In conclusion, two techniques can be employed to obtain gnotobiotic cultures of aquatic organisms, which are sensitive to treatments with disinfectants: (i) carefully remove axenic gametes from killed parents; or (ii) disinfecting non-axenic gametes or larvae. For the latter technique, the following procedures can be used: (i) in sterile conditions, gametes or larvae are initially rinsed with filtered and autoclaved medium to remove as much as possible the contaminants; (ii) the disinfectant or combinations of disinfectants (in low concentrations) is/are applied to kill the remaining micro-organisms; and (iii) the disinfectant(s) and dead microbes are removed from the medium by rinsing the organisms with filtered and autoclaved medium. If these procedures are not sufficient to establish an axenic environment free of live and dead (as much as possible) bacteria, the three steps must be repeated until the task is accomplished. Whenever the gametes obtained with the first technique are contaminated, the second disinfection procedure has to be performed.

The second critical step to culture gnotobiotic aquatic animals is their maintenance. The success of a gnotobiotic culture depends upon the availability of an appropriate apparatus able to simulate the environment found in nature and where animals can live with the lowest disturbance and maximum comfort as possible. Therefore, special isolators or recipients are needed to culture germ-

free organisms, which need to be absolutely impervious to micro-organisms and easily sterilized. Most studies with gnotobiotic aquatic animals used glassware containers easily sterilized by autoclaving, or sterile plastic recipients. To prevent contaminations of the isolator and consequently of the cultured organism, all equipment were previously packaged and sterilized using autoclaving or irradiation. Different sterile feeds were provided depending on the animal group cultured, using preferably axenic live food (e.g. microalgae, rotifers, *Artemia*, nematodes, yeast, bacteria, diatoms), autoclaved or irradiated feed (e.g. yeast, bacteria, microalgae, cerophyl, lactoserum, soybean), or a complete sterile nutritive particulate medium (only in very few studies, mostly crustaceans) (e.g. Provasoli and D'Agostino 1969). All handlings were performed using aseptic techniques (precautionary measures taken to prevent contamination of pure cultures and sterile laboratory equipment) and, in most studies, using a laminar flow hood. Especially when germ-free organisms are transferred for the first time to the isolator, at least some form of quarantine should be applied before starting an experiment.

### Methods to verify gnotobiology

The microbiological monitoring forms an essential part of any investigation involving gnotobiotic organisms, as the results obtained with these tests will determine the status of the animal. Sterility of an organism, i.e. the total absence of foreign viable associates (either virus or bacteria), cannot be assured by absolute criteria. Several reasons explain the complexity to set specific methodologies to check contaminants: (i) the possibility of a pre-existent host contamination in the course of embryonic development; (ii) the microbial invasion of the isolator, due to insufficient sterilization of air, feed, and tools; (iii) and the difficulty to detect certain microbes associated with organisms (Gordon and Pesti 1971). To overcome these limitations, it is fundamental to monitor periodically several samples from different culture levels, like the animal, the feed and the culture medium.

Presently, the complete axenicity of a culture system is difficult to assure, as there is no methodology able to provide in a reproducible manner proof of axenic cultures, as all methodologies present some limitations (e.g. detection limit of bacteria). Studies performed so far with axenic aquatic organisms used two approaches to evaluate contaminants: (i) bacteriological culture media (e.g. marine agar, infusion broth, brain-heart infusion broth), ranging from those routinely used to isolate common micro-organisms to more specialized media suitable for detecting specific micro-organisms more demanding in terms of nutritional requirements; and (ii) microscopic

examination of samples using stained preparations (e.g. 4',6-diamidino-2-phenylindole, DAPI; acridine orange; 5-(4,6-dichlorotriazin-2-yl) aminofluorescein, DTAF; tetrazolium salt, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT), in order to detect microbes that do not grow on artificial media. Only few studies combined both approaches (see Tables 1 and 2) and in all studies no mention was made on the efficiency of the techniques used to check for contaminations. Plating on media is not necessarily an accurate technique, as a considerable amount of bacterial species cannot be enumerated in this way. In addition, the results are only available after a relatively long period (at least 24–48 h). The use of stained preparations (e.g. live or DNA stains) seems to be more accurate and less time consuming than the use of bacteriological media to detect contaminations, as these dyes detect all bacteria present in the culture and the results are available in a shorter period. Nevertheless, stained preparations also present some limitations. Although live staining techniques (e.g. MTT, CFDA, Chemchrom B and BacLight) allow an easy detection of all live, but not dead bacteria, previous findings demonstrated that dead bacteria can dramatically influence the results obtained with gnotobiotic organisms (Marques *et al.* 2005). The use of DNA fluorescent staining techniques (e.g. DAPI combined with SYTOX green nucleic acid stain, which detects live and dead bacterial cells on the basis of plasma membrane integrity) is certainly more accurate than the use of live dyes. DAPI dye the DNA of intact cell membranes, while SYTOX stains DNA of damaged cell membranes (Wierzbos *et al.* 2004).

The complexity of detecting contaminations in gnotobiotic organisms (with a known MC) is even higher and hence more difficult to establish than for axenic organisms. In fact, the presence of any microbe constitutes a contaminant to axenic animals, while in gnotobiotic organisms, it is necessary to differentiate between contaminants and known microflora (Trexler 1978). In axenic organisms a larger spectrum of methodologies can be applied to detect contaminants than in gnotobiotic organisms. In fact, although live and DNA dyes are valuable tools to verify contaminations of axenic organisms, their applicability in experiments performed with gnotobiotic animals (cultured with known microbes) is more complicated, as both the known microbiota and the contaminants will be stained with these dyes. The evaluation and quantification of contaminants in gnotobiotic systems can be potentially performed with specific methodologies, such as molecular biology techniques [e.g. denaturing gradient gel electrophoresis (DGGE) analysis of PCR fragments generated by universal primers analysis of for instance 16S rDNA or 16S rRNA], fluorescent microscopy using fluorescent stains (e.g. DAPI, DTAF, acridine

orange, fluorescent tagged antibodies, ethidium bromide, Hoechst stains, rhodamine, a combination of fluorescent probes: one targeting activity and one targeting membrane integrity), or specific media (if the known microbiota is previously labelled with a fluorescent marker). Nevertheless, studies should be performed to evaluate and validate the potential of such techniques, as they also present some limitations. Although in monoxenic cultures, the amplification of 16S rRNA can be routinely possible starting from one cell (Tsen *et al.* 1998), this detection limit may increase considerably, due to the methodology employed (e.g. normal PCR, nested PCR, real-time PCR) or due to the bacterial strain used (Satokari *et al.* 1998). In addition, in mixed cultures, bacteria that constitute <1% of the population are not detected neither (Muyzer *et al.* 1993), while dead bacteria can be detected (Satokari *et al.* 1998).

Therefore, experiments with gnotobiotic aquatic animals should use a combination of several appropriate techniques (e.g. combinations of culture media with live and/or DNA stains), instead of using single methodologies, to detect contaminations, keeping in mind that even sophisticated technologies, like DGGE, have their own limitations. In addition, axenic control treatments should be always performed in experiments where specific microbes are tested in gnotobiotic animals (to help evaluating contaminations). When the presence of contaminations is demonstrated in an isolator, the results must be discarded.

### Findings with gnotobiotic aquatic animals

The interactions occurring between the host and microbes are extremely complex, as they are influenced by environmental parameters and by the interactions established between microbes. Although gnotobiotic aquatic organisms are potential model systems to study host–microbe interactions, the extrapolation of results to other organisms seems to be very difficult. According to previous studies performed in terrestrial animals by Gerard *et al.* (2004), the dominant species of each bacterial group present in the MC seems to be specific of each animal group. These authors compared the MC of gnotobiotic rats with the human or pig microbiota, and showed that the dominant MCs of gnotobiotic rats, human or pig were similar with regard to the major phylogenetic groups, but distinct when focusing at the bacterial species level. The literature gathered above with gnotobiotic aquatic organisms seems to confirm this trend, as an important diversity of bacteria presenting positive or negative effects was found between the animal groups. Nevertheless, some bacteria presented common beneficial effects to several animal groups, such as: *Alteromonas* sp. (molluscs: Douillet and

Langdon 1993; and rotifers: Douillet 2000a,b); some *Vibrio* sp. (crustaceans: Gomez-Gil *et al.* 1998; Verschuere *et al.* 2000b; and rotifers: Martínez-Díaz *et al.* 2003); *Alcaligenes* sp. and *Bacillus* sp. (molluscs: Erasmus *et al.* 1997; and crustaceans: Verschuere *et al.* 1999, 2000b); *Moraxella* sp. (molluscs: Erasmus *et al.* 1997; crustaceans: Verschuere *et al.* 1999, 2000b; and rotifers: Hagiwara *et al.* 1994); *Aeromonas* sp. (marine fish: Hansen and Olafsen 1989; Munro *et al.* 1995; crustaceans: Verschuere *et al.* 1999, 2000b; and rotifers: Martínez-Díaz *et al.* 2003); *Pseudomonas* sp. (molluscs: Erasmus *et al.* 1997; and rotifers: Hagiwara *et al.* 1994); *Cytophaga* sp., *Roseobacter* sp., *Ruegeria atlantica* and *Paracoccus* sp. (rotifers: Rombaut *et al.* 1999; and crustaceans: Marques *et al.* 2005); thus requiring further research to evaluate possible generalizations. Although for aquatic organisms, specific host–microbe associations are expected to exist, the host gene expression and responses induced by the microbiota seem to be similar in different animal groups. Rawls *et al.* (2004) found that in zebra fish and mouse micro-organisms were able to induce 59 similar responses in their intestine, including those involved in stimulation of epithelial proliferation, promotion of nutrient metabolism, xenobiotic metabolism and innate immune responses. The major findings of host–microbe interactions using gnotobiotic aquatic animals as test organisms are described above.

### Freshwater and marine fish

To our knowledge, Rawls *et al.* (2004) evaluated for the first time the host–microbe interactions using gene expression analysis in gnotobiotic aquatic animals, namely the freshwater zebra fish (*Danio rerio*) (Table 1). The use of gene expression analysis offers a unique opportunity to study the effect of microbes at the genomic level, in order to document the wide range of host functions that are modulated by the microbiota. This study pointed out that microbes regulate the expression of several genes (involved in DNA replication, cell division, lipid metabolism, innate immunity), as well as host responses (e.g. increased epithelial proliferation). Some genes were always expressed (independently of the type of micro-organism used), while the expression of other genes was bacteria specific, suggesting that at least a subset of zebra fish genes is sensitive to unknown factors induced by specific bacteria present in the gut microbiota. This expression differed considerably according to the animal developmental stage. In addition, these authors studied the effects of bacteria in zebra fish morphology. A high morphological similitude was found between axenic, non-axenic fish and axenic animals cultured with the microbiota collected in the culture medium of non-axenic fish until 8 days

postfertilization. Morphological differences between the three fish cultures only occurred at 9 days postfertilization. Axenic animals began to develop a progressive epidermal degeneration phenotype, manifested by epidermal opacity, loss of epidermal integrity, and sloughing of epidermal cells. These phenotypes were rescued by exposing 3 or 6 days postfertilization axenic animals to the microbiota of non-axenic animals. Therefore, the authors postulated that the degenerative changes observed in the late larval stages of axenic animals are likely due to the absence of microbes.

Several studies were performed on host–microbe interactions using gnotobiotic marine fish (Table 1). Hansen and Olafsen (1989) analysed the microbial interactions with cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) using scanning electron microscopy. Unfortunately, the method employed to obtain axenic fish eggs was not described. Marked bacterial growth was observed only 2 h after fertilization and, during hatching, eggs presented heavy bacterial concentrations. Several adherent bacteria were identified in the surface of both cod and halibut eggs (*Pseudomonas* sp., *Alteromonas* sp., *Aeromonas* sp. and *Flavobacterium* sp.), while *Vibrio fischeri* was the only adherent bacterium detected in cod eggs and *Moraxella* sp. and *Alcaligenes* sp. were found only on halibut eggs. The authors also detected the presence of bacteria (*Caulobacter* sp. and *Seliberia* sp.) in ovaries attached to cod eggs before spawning, thus contributing to abolish the dogma that gonads are considered as sterile. In a second set of experiments, the authors attempted to regulate the egg microflora by incubating gnotobiotic eggs with the antibiotic-producing bacterial strains *Pseudomonas* sp. and *Alteromonas* sp., isolated from five green and brown marine microalgae: *Enteromorpha intestinalis*, *Enteromorpha compressa*, *Fucus ceranoides*, *Pelvetia canaliculata* and *Ulva lactuca*. However, the eggs primed with these bacterial strains presented similar colonization to the controls not inoculated with such bacteria, meaning that the antibiotic-producing strains failed to prevent adherence of environmental microbiota. Meanwhile, Munro *et al.* (1995) cultured germ-free turbot (*Scophthalmus maximus*) larvae fed with gnotobiotic rotifers (*Brachionus plicatilis*) supplied with known bacteria. *Vibrio anguillarum* was found to be responsible for significant higher mortalities to fish larvae in comparison with bacteria-free controls, while turbot larvae inoculated with an *Aeromonas* sp. (isolated from a healthy batch of copepod-fed larvae), presented no significant differences in survival compared with the control treatment. Finally, Verner-Jeffreys *et al.* (2003) used bacteria-free halibut (*H. hippoglossus*) larvae to test several bacterial isolates from British halibut hatcheries (*Pseudoalteromonas* sp., *Halomonas marina*, *Vibrio salmonicida*-like,

*Photobacterium phosphoreum* and *Vibrio splendidus*), as well as the pathogenic bacterium *V. anguillarum*. With the exception of *V. anguillarum*, all the remaining bacterial isolates were not harmful to the fish larvae, as the treatments performed with those isolates presented no statistical differences in survival when compared with the bacteria-free controls. In a second set of experiments, three micro-organisms (a *Pseudoalteromonas* strain and two *Carnobacterium*-like strains), previously shown to inhibit growth of pathogenic bacteria *in vitro*, were tested for their ability to protect halibut larvae against *V. anguillarum*. Yet, none of the isolates were able to protect the fish larvae against this pathogen. Although no bacterium was found to protect gnotobiotic marine fish against pathogens, all studies confirmed that marine fish cultured without microbes is an excellent research tool to analyse host–microbe interactions.

### Molluscs

Guillard (1959) carried out preliminary studies regarding mollusc–microbe interactions (Table 1). This author demonstrated that two bacterial strains (*Vibrio* sp. and *Pseudomonas* sp.) isolated from an infected hard clam (*Venus mercenaria*) larva were strong pathogens to healthy larvae cultured in germ-free conditions, while germ-free larvae exposed to those bacteria and simultaneously treated with antibiotics were as healthy as the controls. In a second set of experiments, this author showed that axenic larvae exposed to the culture medium of virulent bacteria (previously filtered or heated to remove all bacterial cells) were able to survive, although presenting a delay in growth. Therefore, it seems that live bacteria, and not the bacterial metabolites present in the culture medium, are essential to damage clam larvae.

Douillet and Langdon (1993) used bacteria-free Pacific oyster larvae (*Crassostrea gigas*) to test the efficiency of several bacterial strains, isolated from the gut of non-axenic adult oysters, to improve oyster survival and growth. Only the strain CA2 (*Alteromonas* sp.) consistently enhanced larval survival and growth in comparison with the bacteria-free control, likely due to a nutritional contribution provided by this bacterium. Unfortunately, the authors did not perform any challenge test with a pathogen to evaluate their hypothesis. Le Deuff *et al.* (1994) and Renault *et al.* (1995) used germ-free *C. gigas* larvae to investigate herpes-like virus (usually associated with high mortalities of oyster larvae in hatcheries). The viral particles were only observed in larvae cultured at 25–26°C, but not at lower temperatures (22–23°C). To evaluate if the temperature or the parental origin of the larvae could influence the expression of the herpes-like virus in *C. gigas*, Le Deuff *et al.* (1996) cultured axenic *C. gigas*

from different parental origins at different temperatures and challenged them with the herpes-like virus. Only the high temperature (and not the parental origin of the larvae) was shown to promote the early production of viral particles in association with high larval mortalities. Probably the high temperature activated the latent viral phase and/or enhanced the rate of the viral reproductive cycle. Meanwhile, Arzul *et al.* (2001) successfully demonstrated the interspecific transmission of the herpes-like virus, by infecting axenic larvae of *C. gigas* and *Crassostrea angulata* with virus obtained from the clam *Ruditapes philippinarum* and from *C. gigas*. These results confirm that concomitant mortalities in the larvae of several bivalve species may occur in private hatcheries and are promoted by intensive rearing conditions. The cross-contamination of herpes-like virus diseases may also occur between bivalves cultured in hatcheries and natural populations.

Finally, Erasmus *et al.* (1997) examined the ability of bacteria found in the digestive tract of abalone (*Haliotis midae*) to produce enzymes that assist in the breakdown of seaweeds. For this purpose germ-free abalone larva was used in the experiments. It was found that both the germ-free abalone and the bacteria (*Pseudomonas* sp., *Flavobacterium* sp., *Alcaligenes* sp., *Vibrio* sp., *Moraxella* sp. and *Bacillus* sp.) were able to degrade the polysaccharides laminarin, carboxymethylcellulose, alginate, agarose and carrageenan. Unfortunately, the authors were not able to provide unequivocal evidences that bacteria are able to assist in the breakdown of complex polysaccharides.

### Crustaceans

Most studies performed so far on host-microbe interactions of crustaceans used germ-free *Artemia* as a test organism (Table 2). Gunther and Catena (1980) examined the interaction between three *Vibrio* species (*Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *V. anguillarum*) and germ-free *Artemia* sp. nauplii using microscopy. It was found that *V. anguillarum* was not able to coat *Artemia* and appeared to have no negative effects to this animal, while the other two bacteria coated the shrimp's body and completely inhibited them from swimming when tested at concentrations above  $10^8$  cells  $\text{ml}^{-1}$ , but not at lower concentrations. This coating required 2 h for *V. alginolyticus* and 8 h for *V. parahaemolyticus*. In this way, the authors demonstrated the possibility of spreading both pathogens to other organisms grown in aquaculture systems, via the live feed *Artemia*. Douillet (1987) determined the effect of unidentified bacteria on germ-free *Artemia* sp. nauplii fed with several diets (*Spirulina* sp., cerophyl, yeast, defatted rice bran, soybean and lactoserum, all at a concentration of 2.5 mg  $10 \text{ ml}^{-1}$ ). None of these diets were able to provide axenic nauplii with all their

nutritional requirements, while the addition of unidentified bacteria previously selected from xenic cultures sustained better *Artemia* performance when fed with the same diets. *Artemia* fed with *Spirulina* presented the best performance when inoculated with the selected microflora, followed by lactoserum and soybean. It seems that there is an essential role of microflora to *Artemia* nutrition, although the study lacks a more detailed evaluation of the origin for such improvement in *Artemia* performance. Rico-Mora and Voltolina (1995) evaluated the effect of five bacterial strains isolated from a diatom (*Skeletonema costatum*) culture (two *Vibrio* sp., a *Flavobacterium* sp., a *Pleisiomonas* sp. and an *Aeromonas* sp.) and of two pathogens (*V. parahaemolyticus* and *V. alginolyticus*) to germ-free *Artemia franciscana* nauplii. The authors confirmed the pathogenicity of both *Vibrio* strains, while the other five bacteria were not detrimental to *Artemia*. Nauplii supplied with *Flavobacterium* sp. and *Pleisiomonas* sp., even performed better than the unchallenged controls. Unfortunately, the authors did not investigate the exact reason for the beneficial effect of *Flavobacterium* sp. and *Pleisiomonas* sp. (e.g. bacteria were used as food or bacteria provided enzymes to *Artemia* that allowed a better digestion of the feed). Gomez-Gil *et al.* (1998) evaluated the bioencapsulation of a pathogenic *V. parahaemolyticus* (strain HL57) and a probiotic *V. alginolyticus* (C7b) in germ-free *A. franciscana* nauplii. The efficiency of *Artemia* nauplii in encapsulating bacteria was demonstrated to be strongly dependent on the type (higher with *V. parahaemolyticus* and lower with *V. alginolyticus*) and status (maximum of  $10^3$  CFU nauplii $^{-1}$  with live bacteria and  $10^5$  CFU nauplii $^{-1}$  with dead bacteria) of bacteria used, and time of exposure (better efficiency between 45 min and 2 h of exposure). Verschuere *et al.* (1999) examined several bacterial strains to improve the nutritional value of a sub-optimal food (unidentified irradiated dry feed) to axenic *Artemia* and to control the associated MC. These authors selected nine bacterial strains (two *Kurtzia* sp., *Bacillus* sp., *Aeromonas hydrophila*, *Moraxella* sp., *Alcaligenes* sp., *Nocardia* sp. and *Vibrio* sp.) based on their beneficial effects to *Artemia* growth and survival. In a second set of experiments, the nine bacterial strains were used to pre-emptively colonize the *Artemia* culture water. The pre-emptive colonization with selected bacteria of *Artemia* cultured under sub-optimal conditions was found to drastically influence the MC developed in the culture water. Verschuere *et al.* (2000b) investigated if those bacterial strains could potentially act as biological control agents against the pathogen *Vibrio proteolyticus*. Some bacteria (e.g. LVS8 – *Vibrio* sp.) were able to provide total protection to *Artemia* cultured under sub-optimal conditions and challenged with *V. proteolyticus*, while other bacteria provided only partial protection to nauplii (e.g. LVS2 – *Bacillus* sp.). Curiously, bacterial strains providing only partial pro-

tection also presented a restricted capacity to colonize *Artemia* in comparison with strains providing total protection. In a second set of experiments, the authors visualized the infection route of *V. proteolyticus* using electron microscopy and found that this pathogen affected *Artemia* microvilli and gut epithelial cells, disrupted epithelial cell junctions and reached the body cavity, where they destroyed cells and tissues. Meanwhile, Orozco-Medina *et al.* (2002) evaluated the effect of *Exiguobacterium* sp. and *Microbacterium* sp. (both strains are usually associated with *Artemia* cysts) in the survival and development of bacteria-free *A. franciscana* larvae cultured under sub-optimal conditions (fed with autoclaved baker's yeast) and challenged with the pathogen *V. parahaemolyticus*. In monoxenic cultures, both *Microbacterium* (strain B) and *V. parahaemolyticus* were found to negatively affect *Artemia*, while the *Microbacterium* (strain A) and the *Exiguobacterium* sp. were harmless, having no impact on survival, growth, and larval development when compared with the bacteria-free control. The challenge tests of *Artemia* using *V. parahaemolyticus* and either *Microbacterium* sp. (strain A) or *Exiguobacterium* sp. resulted in a significant positive effect on the growth and development of *Artemia* larvae in comparison with monoxenic cultures of *Artemia* challenged with *V. parahaemolyticus*. Therefore, both strains can be considered as potential probiotic candidates to *Artemia*. Although the experimental setups employed in the studies performed by Verschuere *et al.* (1999, 2000b) and by Orozco-Medina *et al.* (2002) are very complete and with a wide range of methodologies, the use of animals cultured in sub-optimal conditions instead of animals cultured in better conditions could have negatively influenced the results obtained jeopardizing the reached conclusions. In fact, results obtained by Marques *et al.* (2005) indicate that in combination with poor and medium quality live feeds (yeast or microalgae), dead or live bacteria were found to exert a strong effect on gnotobiotic *Artemia* survival but a rather weak or no effect on individual length. These effects were reduced or even disappeared when medium to good quality major feed sources were used, possibly due to improvements in the health status of *Artemia*. Marques *et al.* (2005) studied the effects of 10 dead or live bacterial strains (eight putative probiotic: *Bacillus* sp., *Aeromonas hydrophila*, *Vibrio* sp., *Cytophaga* sp., *Roseobacter* sp., *Ruegeria atlantica*, *Paracoccus* sp., and an alpha-proteobacteria; and two pathogenic: *Vibrio campbellii* and *V. proteolyticus*) on *Artemia* performance using gnotobiotic *Artemia* cultured under different feed conditions (with the microalga *Dunaliella tertiolecta*, or with a baker's yeast *Saccharomyces cerevisiae*). Some probiotic bacteria, such as GR 8 (*Cytophaga* sp.) improved the nauplii performance beyond the effects observed with dead bacteria, independently of the feed supplied, and thus, this strain was considered as a promising candidate probiont.

Soto-Rodriguez *et al.* (2003) examined the relationship between the mortality of germ-free *A. franciscana* nauplii and various luminous vibrios (mostly *Vibrio harveyi*) obtained from sea water and from diseased shrimps. Significant mortalities were observed with nine bacterial strains inoculated at  $10^5$  to  $10^6$  CFU ml<sup>-1</sup> in comparison with unchallenged nauplii. In addition, the authors demonstrated that *Artemia* could encapsulate a maximum of 10 to  $10^3$  CFU of bacteria per nauplius, without any apparent relationship between the densities of bacteria inoculated, the amount of bacteria ingested, and the naupliar mortality. Therefore, the virulence of the bacterial strains was suggested to be more related to the production of particular exoenzymes than to colonization factors. Defoirdt *et al.* (2005) used gnotobiotic *A. franciscana* as a test system to perform a very interesting study on the impact of mutations occurring in the quorum sensing systems of *Aeromonas hydrophila*, *V. anguillarum* and *V. harveyi* and their virulence. The authors found that mutations in the autoinducer 2 (AI-2) synthase gene *luxS*, the AI-2 receptor gene *luxP* or the response regulator gene *luxO* of the dual channel quorum sensing system of *V. harveyi* abolished virulence of the strain towards *Artemia*. Moreover, the addition of an exogenous source of AI-2 could restore the virulence of an AI-2 nonproducing mutant. In contrast, none of the mutations in either the acylated homoserine lactone-mediated component (AI-1) of the *V. harveyi* system or the quorum sensing systems of *Aeromonas hydrophila* and *V. anguillarum* had an impact on virulence of these bacteria towards *Artemia*. These results seem to indicate that the disruption of quorum sensing could be a good alternative strategy to combat infections caused by *V. harveyi*. Marques *et al.* (2006) evaluated for the first time in a gnotobiotic system the immunostimulatory nature of  $\beta$ -glucans using *Artemia* as a test system. Two strains of baker's yeast (differing in the amounts of  $\beta$ -glucans present in their cell wall) were added in small concentrations to *Artemia*, or a commercial product (consisting of pure glucan particles originating from the cell wall of baker's yeast, being subsequently challenged with one *Vibrio* pathogen: *V. campbellii* or *V. proteolyticus*). It was found that *Artemia* supplemented with small quantities of the yeast strain presenting higher concentrations of  $\beta$ -glucans, or with glucan particles, completely resisted to detrimental effects of both pathogens. The higher amount and/or availability of  $\beta$ -glucans in that yeast strain might play an essential role in such protection, as most probably glucans stimulate the innate immune response of nauplii.

Jung and Hagiwara (2001) investigated the effect of co-existing unidentified bacteria on the interspecific relationship of the copepod *Tigriopus japonicus* with the rotifer *Brachionus rotundiformis* in axenic and synxenic cultures. It was found that the rotifers' growth was sup-

pressed by the presence of *T. japonicus* in axenic cultures, although the copepod could not persist in such particular culture conditions. Yet, copepods performed better when grown in synxenic cultures, likely due to the use of bacteria as feed source, without suppressing the rotifers' growth. These results suggest that bacteria are able to modify the interspecific relationship between species, although the exact mechanism was unfortunately not studied in detail.

### Rotifers

Meadow and Barrows (1971) performed preliminary studies on host–microbe interactions of germ-free rotifers (Table 2), by using the germ-free rotifer *Philodina acuticornis* to test several bacteria and microalgae as feed sources. Optimal reproductive rates and longevity of rotifers were obtained with a bacterial mixture of *Aerobacter aerogenes* and *Pseudomonas* sp., while the reproductive rate was reduced in animals cultured under monoxenic conditions with *Aerobacter aerogenes*, with *Pseudomonas* sp., with the microalga *Chlorella vulgaris* or with the microalga *Chlamydomonas reinhardtii*. This interesting study highlights the complexity of interactions that can exist between hosts and microbes. Sometimes only the combination of several bacteria is able to significantly improve the host performance, while the use of a single bacterial strain does not affect the host in any way. Unfortunately, the authors were not able to find the reasons for the beneficial effect provided by the bacterial mixture.

Hagiwara et al. (1994) studied the dietary effect of several bacteria to the sexual and asexual reproduction of the bacteria-free rotifer *B. plicatilis*. It was found that the sexual reproduction rates and resting egg formation were 4–10 times higher when provided with three bacterial strains (*Pseudomonas* sp., *Moraxella* sp. and *Micrococcus* sp.) than in the bacteria-free controls. Similarly, an improvement in the sexual reproduction rates and resting egg formation was observed with the addition of water-soluble extracts from non-axenic rotifers (previously filtered to remove all bacteria). This seems to indicate that some unidentified water-soluble substances released by bacteria are able to induce an enhanced sexual reproduction of rotifer. Unfortunately, these substances were not identified by the authors. Such substances can potentially be used in aquaculture to better control the life cycle of rotifers that are used as feed to fish and shellfish larvae. Douillet (1998) evaluated the effect of unidentified bacteria isolated from resting eggs of non-axenic rotifers *B. plicatilis* and *B. rotundiformis* in the hatching efficiency of germ-free animals. The unidentified microbiota present in the resting eggs was found not to produce any adverse effect on rotifers' hatching efficiency. Meanwhile, Douillet

(2000a) evaluated the effects of several marine microbes or bacterial additives on the growth rate of axenic *B. plicatilis* fed with a sterile artificial diet (microfine *Spirulina* and torula dried yeast) or with microalgae (*Isochrysis galbana* or *Chlorella minutissima*). The highest improvement in rotifer growth rate was obtained with four bacteria (an *Alteromonas* strain and three unidentified Gram-negative strains B3, B4 and B5) in all feeding regimes. In a second set of experiments, Douillet (2000b) cultured germ-free *B. plicatilis* and *B. rotundiformis* fed with an artificial diet (microfine *Spirulina* and torula dried yeast) and inoculated with combinations of the four bacteria presenting beneficial effects in the previous study. For both rotifer species, the highest growth rates and the lowest variation between replicates were obtained with the *Alteromonas* strain or with the mixture of bacterial strains composed mainly by *Alteromonas* sp. The author revealed that the supplement of bacterial mixtures, all beneficial for rotifers, and differing only in the proportion of their constituents led to significantly different results, thus demonstrating the complexity of microbial ecology processes. Meanwhile, Rombaut et al. (1999) examined the effect of several bacterial strains (isolated from well-performing live-feed production systems), in the growth rate of monoxenic *B. plicatilis*. Only nine strains were able to significantly improve the rotifer growth rate and the average amount of amictic eggs per rotifer (*Cytophaga* sp., *Roseobacter* sp., *Ruegeria atlantica*, *Paracoccus* sp., *Aeromonas hydrophila*, *Moraxella* sp. and three unidentified bacterial isolates) in comparison with bacteria-free animals. Although the authors were able to demonstrate that it is possible to control the MC in rotifer cultures by adding bacterial strains with a positive effect on the population growth rate, unfortunately the mechanism involved in such effect was not explored. Martínez-Díaz et al. (2003) evaluated the encapsulation of live bacteria in the monoxenic rotifer *B. plicatilis*, and demonstrated the successful incorporation of the tested bacteria (*V. harveyi* and *Vibrio carchariae*). It was found that the rotifers' performance (survival and growth rates) were, in most cases, not affected by the presence of several Vibrionaceae species (*V. carchariae*, *V. campbellii*, *V. parahaemolyticus* and *Aeromonas ichtiosmia*), while significant improvements in rotifer growth rates and even amictic egg formation occurred with *Vibrio proteolyticus* and *Aeromonas media*. Regrettably, once again the mechanisms involved in the beneficial effects provided by *Vibrio proteolyticus* and *Aeromonas media* were not investigated. Jung and Hagiwara (2001) investigated the effect of co-existing aquatic bacteria on the interspecific relationship between the axenic rotifer *B. rotundiformis* and the axenic copepod *Tigriopus japonicus*. The growth of *B. rotundiformis* was always suppressed by the presence of *T. japonicus* in axenic cultures, but not in cultures performed

with a selected unidentified bacterium. This result suggests that the presence or absence of specific bacteria in the aquatic environment might alter the relationship between species of aquatic animals and between these organisms and their environment. Unfortunately, the mechanisms that allow the unidentified bacteria to interact with the relationship between different aquatic animal species were not studied in detail. Tinh *et al.* (2006) tested the effect of MCs isolated from either normal-performing or crashed rotifer cultures in gnotobiotic rotifers *B. plicatilis* fed with three different food types, i.e. *Chlorella* sp., the wild-type baker's yeast (*Saccharomyces cerevisiae*) and a baker's yeast mutant, which is deficient in cell wall-bound mannoprotein. Interestingly, in the presence of all live MCs (including the one originating from a crashed rotifer culture), rotifers fed with both yeasts strains as major feed sources (but not with the microalga) presented significantly higher rotifer growth rate than rotifers cultured with the same feed sources in the absence of MCs or with heat-killed MCs. The results of this interesting study demonstrate that gnotobiotic rotifer cultures obtained from axenic amictic eggs can be used as a test system for studying microbial-attributed as well as nutritional functions in the aquatic food chain. Unfortunately, this study lacks an identification of the bacteria present in each MC tested (this important information will be very useful for upcoming studies).

### Cnidarians

Rahat and Diementman (1982) cultured bacteria-free *Hydra viridis* (symbiotic and apo-symbiotic) and *Hydra vulgaris* (nonsymbiotic), using axenic nauplii of *Artemia salina* as feed (Table 2). Under bacteria-free conditions the nonsymbiotic hydra did not form any bud, while the same hydra fed with nonsterile *Artemia* or inoculated with unidentified bacteria (isolated from budding stock cultures) were able to resume budding. These results suggest that an exogenous budding factor (provided by nonsterile *Artemia* or by some bacteria), such as nutrients, vitamins or hormones, was required by the nonsymbiotic hydra for its vegetative reproduction. This budding factor was suggested to be endogenous in symbiotic and apo-symbiotic *H. viridis*, as these animals obtained all required nutrients from bacteria-free *Artemia* to form buds. More experiments are still required to evaluate what is the exact exogenous budding factor required by nonsymbiotic hydra.

### Conclusions

The major conclusions of the present synopsis regarding the use of gnotobiotic aquatic animals to study host-microbe interactions are:

- i There is a huge diversity of disinfection/sterilization methods to produce axenic organisms. Therefore, the standardization of the disinfection procedures is proposed according to the developmental stage of the animal to be axenised, by rinsing the animals with sterile medium (filtered and autoclaved) before and after using disinfectants.
- ii There is also an enormous diversity of methodologies used to verify gnotobiology, as no methodology is able to provide in a reproducible manner proof of axenic cultures due to their intrinsic limitations. In this way, the use of a combination of several appropriate techniques (e.g. culture media with live and/or DNA stains) is proposed to detect contaminations, instead of using single methodologies. In addition, axenic control treatments should be always performed in experiments where specific bacteria are tested in gnotobiotic animals (to help evaluate contaminations).
- iii According to literature gathered in the present synopsis, specific host-microbe associations are expected to exist in aquatic animals (e.g. production of enzymes that assist in the breakdown of nutrients, production of antimicrobial substances, bacterial adherence, modification of the interspecific relationship between hosts, releasing water-soluble substances that affect the host, influencing the host reproductive rate), while the host gene expression and other responses induced by the microbiota seem to be similar in different animal groups (e.g. pathogenicity, stimulation of epithelial proliferation, promotion of nutrient metabolism, xenobiotic metabolism and innate immune responses).
- iv Therefore, the use of gnotobiotic aquatic animals is an excellent tool to extent the understanding of the mechanisms involved in host-microbe interactions and to evaluate new treatments of disease control.
- v Future studies of host-microbe interactions using gnotobiotic aquatic animals should consider all possible interactions, such as the performance of the host, the quantitative and qualitative evolution of the microflora, and the differential gene expression.

### Future research

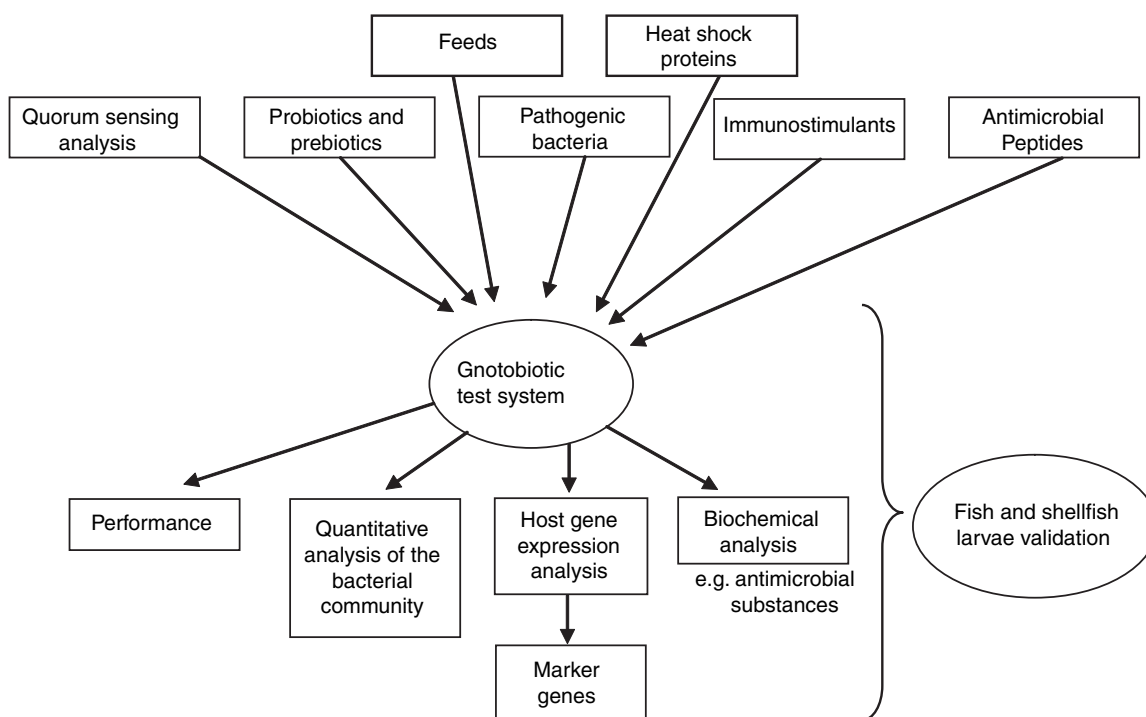
Although gnotobiology is an old science, technological advances allowed the development of new methodologies (e.g. gene expression analysis, genetic manipulation of host and colonizers). The combination of these methodologies with gnotobiology will certainly provide better and more reproducible insights related with several issues in aquatic organisms, such as: (i) host-microbe interactions (e.g. factors involved in host colonization by bacteria, ontogeny of the gastrointestinal tract, tissue repair, senescence of cells and tissues, modulation of innate or adaptive immunological defence mechanisms, infection

route and pathogenicity of bacteria); (ii) test new treatments of disease control (e.g. probiotics, immunostimulants, antimicrobial peptides); (iii) provide important information (e.g. nutritional requirements, ontogenic development of endogenous enzymes in larval digestive systems); (iv) study the adaptive and/or innate immune system; (v) evaluate the effect of toxic materials or radiations in contaminated organisms. Nevertheless, no single gnotobiotic model organism or methodology will, by itself, provide a complete picture of these processes.

Future studies of host–microbe interactions using gnotobiotic aquatic animals should consider all possible interactions, e.g. the performance of the host (e.g. survival, growth or histological development) as a function of the quantitative evolution of the microflora (in the culture medium, as well as in the animal), and as a function of the differential gene expression (see Fig. 1). Coupling gnotobiotic aquatic organisms with quantitative functional genomics (e.g. gene expression analysis) can be a valuable tool to document the wide range of host functions that are modulated by the analysed factor (e.g. microbiota, immunostimulants), to identify the host and factor signals and to signal pathways that mediate such interactions. The combination of both methodologies has been successfully employed by several authors using gnotobiotic aquatic (zebra fish; Rawls *et al.* 2004) and terrestrial (mice; Xu and Gordon 2003; Gerard *et al.* 2004) animals. However, these studies should not be confined

to the gene level, but should also consider each of the steps that progress from gene expression (transcription) through transactional and post-transactional processing to the release of mature and active proteins. The identification of genes (through gene expression analysis) and biochemical pathways involved in the promotion of a specific factor (e.g. immunity, growth, survival) may be useful in the long term to monitor the health status of cultured animals as preventive measures at all stages of their development, as well as to genetically select animals for specific criteria (e.g. disease resistance). This tool will be extremely useful for a sustainable aquaculture production.

Studies using standardized gnotobiotic aquatic organisms are usually performed with larval and juvenile stages. However, they should be extended to adult stages and validated on a wide range of target organisms. The biological effects of, e.g. host–microbe interactions or new treatments of disease control should be as well evaluated on the host over long-term use. To perform such refined studies, the gnotobiotic husbandry protocols of aquatic animals will require many more improvements, such as: identification and standardization of diets that allow the survival of gnotobiotic organisms to reproductive maturity; the capacity to breed strains of germ-free aquatic animals for a complete life cycle or even over multiple generations; and creating efficient equipment able to provide complete control of the environmental variables.



**Figure 1** Schematic representation of future research with gnotobiotic aquatic animals.

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