# Mariculture Systems, Integrated Land-Based

Muki Shpigel
Israel Oceanographic and Limnological Research, National Center for Mariculture, Eilat, Israel

## Article Outline



## Glossary

**Detritivores** (also known as *saprophages*) They are heterotrophs that obtain nutrients by consuming detritus (decomposing organic matter).

**Halophyte** Salt-loving plants that can be grown at higher salinities than most traditional crop plants.

**IMTA** The Integrated Multi-Trophic Aquaculture System (IMTA) is an aquaculture practice in which excretions of one or more organisms are utilized by other cultured organisms from different trophic (nutritional) levels within the system.

**Land-based and offshore mariculture systems** Two methods of seawater aquaculture (mariculture); the former on land and the latter in the ocean.

**Polyculture** An aquaculture practice which involves culture of two or more species from the same or different trophic levels in the same water reservoir.

**RAS** Recirculated Aquaculture System (RAS) is an aquaculture practice for the rearing of aquatic organisms wherein 90% or more of the water is recycled within the system.

**Sludge** Solid/particulate waste that includes, among other components, feces, uneaten feed, algae and bacteria, which sinks to the bottom of aquaculture water reservoirs.

## Definition of Subject

The Integrated Multi-Trophic Aquaculture System (IMTA) is an aquaculture practice in which excretions of one or more organisms are utilized by other cultured organisms from different trophic (nutritional) levels. IMTA systems are distinct from polyculture systems, which involve two or more species from the same or different trophic levels in the same water reservoir. In a typical IMTA, the various species are cultured in separate spatial entities, permitting intensification and optimization of production. The IMTA concept has been increasingly adopted in modern day aquaculture, including land-based (Fig. 1) [1–5] and offshore mariculture [6, 7].

In land-based IMTA systems, seawater is pumped from the sea to fish or shrimp ponds. A pelleted diet is the only source of nutrients for the animals in the system. Nutrient-rich effluent water from these ponds can take three directions: microalgae ponds, macroalgae ponds, and constructed wetlands with halophyte plants. The microalgae can be utilized by filter feeders such as *Artemia* or/and bivalves. The macroalgae can be utilized by macroalgivores such as abalone or sea urchins, and detritus can be utilized by detritivores such as mullets, sea cucumbers, or polychaete worms (Fig. 1).

## Introduction

The concept of polyculture and IMTA systems is not new. Such systems of different species of fish, or combinations of invertebrates and fish, have been existing in ancient Egypt and China for thousands of years. Artificial enclosures or natural ponds in tidal zones were generally used. Extensive traditional IMTA and polyculture systems are still practiced today in various parts of Asia in fresh and salt water. Rice and fish are cultured together in China. Earthen ponds, in association with wild or agricultural plants, are used on a wide scale in fish and shrimp farming in China, Indonesia, Taiwan, Thailand, Japan, Vietnam, India, the Philippines, and Ecuador. In Europe, ducks, fish, and crayfish have been raised together in freshwater ponds. This type of extensive production has proven sustainable, because it utilizes organisms that feed on different levels of the food web, and maintains a clean environment.

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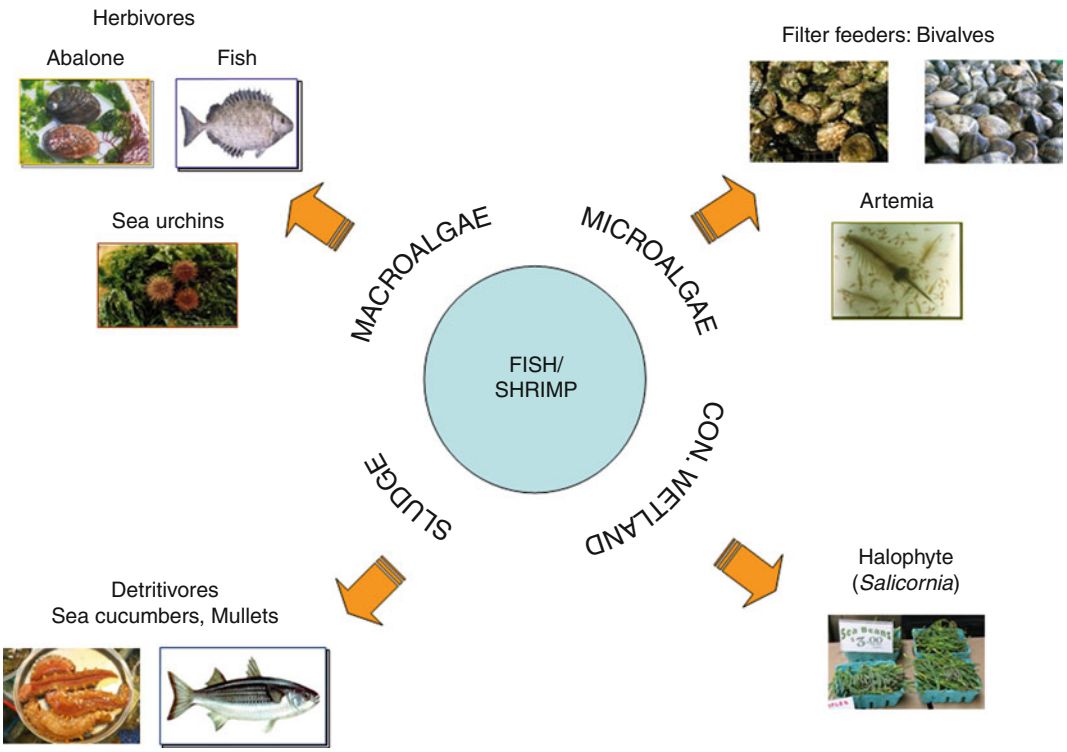



**Mariculture Systems, Integrated Land-Based. Figure 1**
Schematic design of land-based IMTA systems (con. = constructed)

The traditional IMTA and polyculture systems are more environmentally friendly than modern intensive mono-aquaculture systems. These systems utilize fewer resources and do not pollute surrounding waters with waste products, because they generally sustain relatively low stocking densities and do not employ fertilizers. Most of them rely on natural production of food. This concept has increasingly been adopted for modern aquaculture, including land-based and sea-cage mariculture. With dramatic increases in global human population, food demand, and overfishing problems, traditional extensive aquaculture cannot satisfy present demand, and much less so the projected future demand, for sea products.

Modern intensive monoculture systems require high levels of resources and produce undesirable wastes. They are dedicated to a few expensive species and do not generate a large amount of food. Intensive aquaculture uses extensive amounts of resources such as water, feeds, fertilizers, chemicals, and energy, while discharging fecal material, uneaten feed, excretions, and drugs into the environment. In turn, this creates eutrophication of the water, has deleterious effects on marine life, increases the risks of antibiotic resistance in organisms, has an adverse effect on biodiversity, and contributes to habitat destruction. The economic success of intensive monoculture in sea cages or land-based facilities has much to do with the fact that, even today, pollution of the environment involves little or no monetary outlay or penalty for the growers. In most countries, aquaculture does not yet include the cost of effluent treatment. However, in the industrialized nations, this age is coming to a timely end and in Europe, there are already laws and regulations requiring effluent treatment and imposing fines for noncompliance. In some countries, this cost can be as high as €0.5–1 $kg^{-1}$ feed, resulting in an expense of €250,000–350,000 per annum for medium-scale RAS (Recirculating Aquaculture System) farms (250 t/year). Awareness is growing among scientists, industry, the public, and politicians that technologies disregarding environmental impact are neither sustainable nor acceptable.

## History

The development of modern land-based IMTA using extractive organisms such as shellfish, microalgae, and seaweeds began in the 1970s with the pioneer work of Goldman et al. [1] and Ryther et al. [2] in the treatment of household effluents. Phytoplankton was cultured in a mixture of domestic wastewater effluent and seawater, fed to suspension-feeder molluscs, and the dissolved remnants of nutrients in the final effluent were assimilated by seaweeds. As the food value of organisms grown on human waste effluents was questionable, adaptations of this principle to the treatment of intensive aquaculture effluents in both inland and coastal areas were proposed [8] and were followed by the integration into a system of carnivorous fish and abalone (e.g., [9]). The first practical and quantitative integrated land-based cultures of marine fish and shellfish, with phytoplankton as biofilter and food for shellfish, were constructed in Israel by Hughes-Games [10] and Gordin et al. [11]. A semi-intensive seabream and gray mullet pond system with silicate-rich green water, located on the coast of the Gulf of Eilat (Red Sea), supported dense populations of diatoms, excellent for feeding oysters [12, 13]. Later, the development of a practical intensive culture of bivalves in phytoplankton-rich effluents was described in a series of articles [3, 14–18]. Lefebvre et al. [19] showed that detritical waste from intensive fish farming can contribute to the growth of bivalves and reduce particulate matter in the water. Jones et al. [20], using the Sydney rock oyster *Saccostrea commercialis*, significantly reduced the concentration of suspended particulates including algae, bacteria, and inorganic particles in integrated systems.

Studies showing the performance of seaweed in land-based IMTA, initially at laboratory scale and later expanded to outdoor pilot scale, began to appear in the 1970s [21, 22]. The theoretical and practical principles of intensive large-scale land-based seaweed culture were studied and developed first at Woods Hole and later at Harbor Branch Oceanographic Institution in Florida – U.S.A. [8, 23–25]. The quantitative aspects of their functioning have been described [14, 16, 26–29]. Fish, abalone, and seaweed IMTA systems were studied by Shpigel et al. [30], Butterworth [31], and Nobre et al. [32]. The aspects of bioeconomics of land-based IMTA are described by Nobre et al. [32], Neori et al. [33], and Bunting and Shpigel [34].

Offshore IMTA system is a relatively new concept that started in the late nineties and is a modification of the land-based IMTA. In coastal integrated mariculture, shellfish and seaweed are cultured in proximity to cage fish culture [6, 7]. Kelp (brown algae) [35, 36] and red algae [37, 38] efficiently take up dissolved inorganic nitrogen excreted by the fish [39], so that seaweed production and quality are often higher in areas surrounding fish cages than elsewhere [6, 40–42]. However, nutrient removal efficiency in offshore IMTA is still relatively low, ranging between 15% and 25% [43].

The concept of IMTA systems is generic and can be applied to cold, warm, and temperate waters, in intensive, semi-intensive, and extensive systems, in sea cages or land-based facilities, in fresh water in land-based facilities or lakes, and all of the above in closed, semi-closed, or flow-through systems.

In recent years, several enterprises and research facilities have begun setting up land-based IMTA; most of the systems are pilot scale or R&D facilities. The IMTA typically include two or three species. In most of the studies, seaweed and microalgae are used as biofilters for the dissolved nutrients (review by Neori et al. [33] and Soto [44]). A broad spectrum list of selected organisms being used in farms and in R&D is presented in Fig. 2. Key species in cold water are salmon, mussels, and the seaweeds *Gracilaria, Laminaria*, and *Porphyra*. For temperate and warm seawater, sea bream, sea bass, oysters, clams, and *Ulva lactuca* are the predominant cultured species (Fig. 2).

Over 200 species are currently the object of R&D projects and in commercial farms and research institutes around the world, in various climate conditions. A significant number of fish and shellfish are cultured in temperate water, and a relatively low number of fish and large number of seaweeds in cold-water climates (Fig. 3).

## Nutrient Budget in Land-Based IMTA

Protein in fish or shrimp feed is the most expensive component of nitrogen input into the IMTA systems. In conventional cages or ponds, fish or shrimps assimilate only 20–30% of the nitrogen, while the rest

KEY SPECIES CULTURED IN POLYCULTURE AND MULTI-TROPHIC SYSTEMS

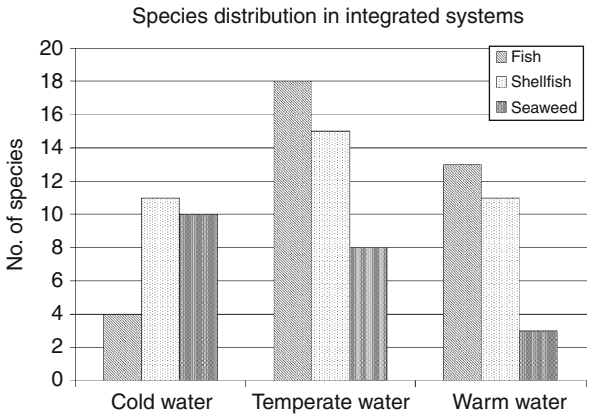
| Cold Seawater | Temperate Seawater | Warm Seawater |
|---|---|---|
| *Oncorhynchus* sp. | *Pagrus major* | *Sparus aurata* |
| *Crassostrea* sp. | *Sparus aurata* | *Lates calcarifer* |
| *Mytilus edulis* | *Dicentrarchus labrax* | *Mugil cephalus* |
| *Haliotis rufescens* | *Mercenaria mercenaria* | *Penaeus* sp. |
| *Gracilaria* sp. | *Ostrea edulis* | *Crassostrea gigas* |
| *Laminaria* sp. | *Ruditapes decussates* | *Tapes japonica* |
| *Macrocystis* sp. | *Gracilaria* sp. | *Haliotis diversicolor* |
| *Porphyra* sp. | *Ulva* sp. | *Gracilaria changii* |
| | *Penaeus* sp. | *Ulva lactuca* |
| | *Crassostrea* sp. | |

| Temperate Brackish Water | Temperate Freshwater | Warm Freshwater |
|---|---|---|
| *Oreochromis* sp. | *Hypophthalmichthys* sp. | *Clarias* sp. |
| *Mugil cephalus* | *Macrobrachium rosenbergii* | *Cyprinus carpio* |
| *Sparus aurata* | | *Oreochromis* sp. |
| *Dicentrarchus labrax* | | *Mugil cephalus* |
| *Penaeus monodon* | | *Penaeus* sp. |

**Mariculture Systems, Integrated Land-Based. Figure 2**
Key species cultured in IMTA and polyculture systems for marine and freshwater environment

is excreted into the water, mainly as dissolved ammonia, feces, and uneaten feed.

Two main practical approaches are emerging for handling the organic and nitrogenous wastes: bacterial dissimilation into gasses in "Recirculating Aquaculture Systems" (RAS), or plant assimilation into biomass (IMTA). Bacterial biofilters are dissimilative. Through a process of nitrification followed by denitrification, bacteria break down the organic pollutants into $N_2$ and $CO_2$ gasses. Bacterial biofilters are technically rather effective for aquaculture and allow significant water recirculation. However, the technology is relatively expensive, and not simple. Bacterial biofilter technologies are suitable for relatively small intensive land-based culture of lucrative organisms. There are no suggestions as to how such technologies can be integrated with large-scale, low cost fish or shrimp production. In addition, this system wastes expensive nitrogen by converting this valuable resource into gas, which is lost into the atmosphere.




**Mariculture Systems, Integrated Land-Based. Figure 3**
Fish, shellfish, and seaweed species combination in IMTA systems in different bio-geographical regions around the world

Nutrient assimilation by other organisms is a more promising method of water treatment. In land-based IMTA ponds, seawater is pumped from the "nuclear species" (fish or shrimp) into the ponds/tanks of secondary organisms or macro-/microalgae. A pellet diet is the only source of nutrients for the primary animals in the system. Nutrient-rich effluent water from these ponds can take three directions: microalgae ponds, macroalgae ponds, or to irrigate halophyte crops (e.g., *Salicornia* sp.). The microalgae can be utilized by filter

feeders such as artemia or bivalves. The macroalgae can be utilized by macroalgivores such as abalone, sea urchins, or herbivorous fish. Halophytes such as *Salicornia* can be used as a food product. The remaining detritus can be fed to detritivores such as mullets, sea cucumbers, or polychaete worms, singly or in combination.

Optimization of the IMTA is typically based on the highest value "nuclear" product at any given time. This "nuclear" product may be shifted according to climatic conditions and economic considerations. For example, in a fish-abalone-seaweed integrated system, abalone is the most valuable species, and the entire system is centered around this species. Abalone will be the first organism to receive the incoming water. From the abalone, the water will drain to the ammonia producers and from there to the biofilters.

The biological and chemical processes in the IMTA system should be balanced between nutrient production by the main organism and nutrient uptake capacity of the micro- and/or macroalgae and downstream by the micro- and macroalgivores. In such systems evaluated in Eilat, Israel, macro-and microalgae were able to assimilate 1–5 g N $m^{-2}$ $day^{-1}$, while algivores and filter feeders assimilated 0.5–1 g N kg $(WW)^{-1}$ $day^{-1}$ (Table 1 and references therein). However, there will be variation in nutrient uptake depending on season and climate, as algal biomass is influenced by day length (i.e., light hours), water temperature, and the nutrient levels in the water.

For example, in a fish-bivalve-seaweed IMTA system in Eilat, 63% of the nitrogen from the feed was assimilated by edible organisms, 32% sank to the bottom as biodeposit (sludge), and only 4.1% was discharged back to the sea (Fig. 4) [3].

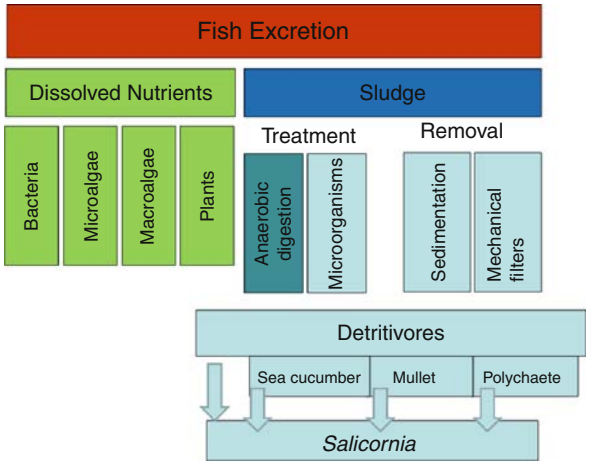
Nitrogen, phosphate, and silicate ratios can vary according to local farm conditions.

Nutrient composition is also affected by additional biochemical processes in the effluent water such as nitrification, denitrification, and ammonification which occurs in the sedimentation pond as well in the pond walls and in the water pipes. These processes can be accelerated or affected by water temperature, nutrient loads, flow rates, and fish feed biochemical composition. Local natural microfauna in the ponds (e.g., zooplankton) and microflora, as well as bloom and crash phenomena, can affect the water quality as well. In most cases, effluent water from fishponds is characterized by a mixture of ammonia, nitrate, and nitrite.

**Mariculture Systems, Integrated Land-Based. Table 1** Assimilation rates of the uptake organisms in land-based IMTA in Eilat, Israel

| | Assimilation rates | References |
|---|---|---|
| Microalgae | 1–3 g N $m^{-2}$ $day^{-1}$ | Shpigel and Blaylock (1991) |
| | | Shpigel et al. (1993a) |
| | | Shpigel et al. (2007) |
| Macroalgae/ *Salicornia* | 3–5 g N $m^{-2}$ $day^{-1}$ | Neori et al. (1991) |
| | | Boarder and Shpigel (2001) |
| | | Schuenhoff et al. (2003) |
| | | Neori et al. (2004) |
| Bivalves/ Artemia | 0.3 g N $kg^{-1}$ $day^{-1}$ 6 g N $kg^{-1}$ $m^{-3}$ $day^{-1}$ (20 kg $m^{-3}$) | Shpigel and Blaylock (1992) |
| | | Shpigel et al. (1993a,b, 1994, 1996) |
| | | Zmora and Shpigel (2006) |
| | | Neori et al. (2004, 2006) |
| Abalone/sea urchins | 0.5 g N kg WW $day^{-1}$ | Shpigel et al. (1996, 1999, 2005, 2006) |
| | | Neori et al. (2001) |
| | | Stuart and Shpigel (2009) |
| *Salicornia* wetland | 2–5 g N $m^{-2}$ $day^{-1}$ | Envirophyte (2010) |
| | | Stuart and Shpigel (2009) |

While macro- and microalgae have proven effective components in land-based systems, neither removes 100% of the dissolved matter and they do not remove particulate matter at all. The remaining waste that includes, among other components, feces, uneaten feed, algae and bacteria, sinks to the bottom and becomes what is known as sludge. This sludge contains valuable ingredients, but can also be toxic to the cultured organisms. It can increase stress and




**Mariculture Systems, Integrated Land-Based. Figure 4** Different pathways to treat sludge from fishponds

disease risk, and reduce the quality of the water both in situ and for reuse. Ignoring the negative effects of the sludge can thus create serious problems and cause financial losses to the farmers. Removing and dumping sludge into the environment would similarly cause damage, even if moderated by dilution, and "foul the fish farmer's own nest" should he use seawater pumped in from the same area. Using detritivores is a novel option for land-based IMTA. Detritivore organisms such as mullets, cockles, and sea cucumbers will assimilate the waste into their bodies, thereby generating a significant saving in treatment costs, while additionally serving as valuable products in their own right, without requiring the purchase of feed for their culture.

The halophyte *Salicornia* sp. as a biofilter in constructed wetlands was evaluated in the "Genesis" and "Envirophyte" EU projects [34, 45, 46]. Using constructed wetlands (CW) planted with halophytes, which would take up the nutrient-rich wastewater and convert it into valuable plant biomass, is a new option for land-based IMTA. This system was developed to a practical stage for cold (UK) and warm (Israel) water. It was found that CW is efficient in clearing water of nutrients and suspended solids, some materials being purified through incorporation into the plants' biomass and others attaching to the substrate or being broken down by bacteria living therein. CW has the benefit of being low cost, is simple to operate, and can be given an aesthetically pleasing appearance. These plants have commercial value as a health food and are potential candidates for the health, beauty, and nutraceutical industries.

## Pilot Scale Systems

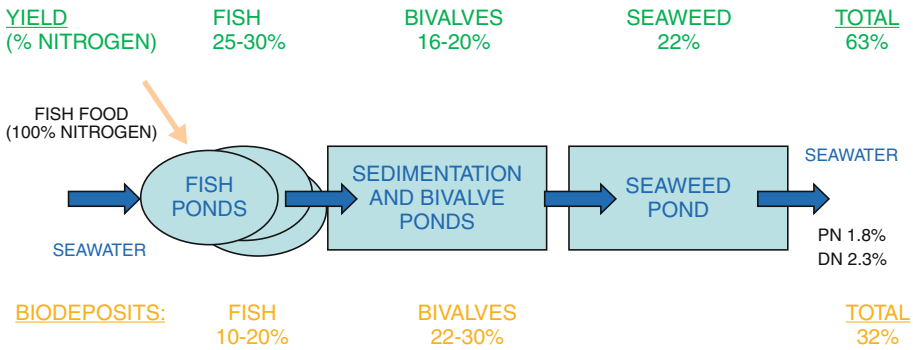
In R&D projects in Eilat, Israel, three different types of IMTA systems were developed:

1. Fish (seabream *Sparus aurata*) – seaweed (*Ulva lactuca*)
2. Fish (seabream *Sparus aurata*) – abalone (*Haliotis discus hannai*)/sea urchin (*Paracentrotus lividus*) (macroalgivores) – seaweed (*Ulva lactuca*)
3. Fish (seabream *Sparus aurata*) – bivalve (*Crassostrea gigas* and *Tapes philippinarum*) – seaweed (*Ulva lactuca*)

In the seabream-*Ulva* system, a daily ration of 1.3 t of feed supported 250 t of fish. This amount of food is equivalent to 64 kg of nitrogen. The fish assimilate around 16 kg of nitrogen. About 9.6 kg of the nitrogen is drained as particulate nitrogen, and 38.4 kg is drained as dissolved nitrogen. One hectare (ha) of macroalgae (*Ulva lactuca*) is required to remove most of the dissolved nitrogen from the water. This system using 500 t of food per year would require an area of 3.4 ha, at a ratio of 1 ha fish to 2.5 ha *Ulva*. Expected yield is approximately 220 t of fish and 1,600 t of *Ulva* (modified from [5] and [47]) (Table 2).

In the seabream-*Ulva*-macroalgivores (sea urchins/abalone) IMTA system, 1 ha of macroalgae produces 1,600 t of *Ulva* annually. This *Ulva* supports 133 t (WW) of abalone (*Haliotis discus hannai*) or 200 t of sea urchins (*Paracentrotus lividus*). A seabream-*Ulva*-sea urchins/abalone IMTA system in Eilat, Israel, using 500 t of food per year will need an area of 5.3 ha, at a ratio of 1 ha for fish, 2.5 ha for *Ulva*, and 1.8 ha for the macroalgivores (modified from [5] and [47]) (Table 2).

In the seabream, microalgae, and bivalves (*Crassostrea gigas* and *Tapes philippinarum*) IMTA system, a daily ration of 1.3 t of feed supports 250 t of fish. The fish assimilate around 16 kg of nitrogen; 38.4 kg of nitrogen is drained as dissolved nitrogen. This system using 500 t of food per year would need an area of 2 ha of phytoplankton pond (with assimilation efficiency of 1–2 g N $m^{-2}$ $day^{-1}$) to support




**Mariculture Systems, Integrated Land-Based. Figure 5**
Nitrogen budget of fish-bivalve-seaweed IMTA system in Eilat, Israel

**Mariculture Systems, Integrated Land-Based. Table 2** Expected performance of land-based IMTA (WW = wet weight)

| IMTA system | Organism | Pond size Ratio/ha | Yield (WW t year$^{-1}$) | Yield (kg WW m$^{-2}$ year$^{-1}$) |
|---|---|---|---|---|
| Fish-*Ulva* (500 t feed y$^{-1}$) | Seabream | 1 | 220 | 22 |
| | *Ulva* | 2.5 | 1,600 | 64 |
| | Total | | 1,820 | 86 |
| Fish-*Ulva* abalone/sea urchin (500 t feed y$^{-1}$) | Seabream | 1 | 220 | 22 |
| | *Ulva* | 2.5 | 1,600 | 64 |
| | Abalone | 1.8 | 185 | 10 |
| | Sea urchins | 1.8 | 140 | 8 |
| | Total | | 1960–2005 | 94 |
| Fish-*Ulva*-clam/oyster (500 t feed y$^{-1}$) | Seabream | 1 | 220 | 20 |
| | Clams/*oysters* | 4 | 140 | 8 |
| | *Ulva* | 0.5 | 70 | 64 |
| | Total | | 430 | 92 |

production of 140 t bivalves and 70 t of seaweed (modified from [5] and [47]) (Table 2).

The economics of these types of land-based IMTA systems were summarized in [5]. However, the economics of a land-based IMTA are site specific since they depend on variables including local construction and operating costs and market prices for the farm's products at any given time [34].

Additional anticipated parameters based on the same model of using 500 t feed per year in each of the three IMTA systems tested in Eilat, Israel, with the projected yields as depicted in Table 2, can be seen in Table 3.

## Future Directions: Challenges and Constraints

Although considerable information is already available for putting land-based IMTA systems into practice, much of it is designed around commercial exploitation of a few high value species that are not affordable for the masses. The challenge for the future is to produce a large quantity of aquaculture products that will be cost-effective for producers, at a reasonable price for consumers, and ecologically sustainable.

Additional studies are required to overcome further constraints, including biological, engineering, and economical aspects:

**Mariculture Systems, Integrated Land-Based. Table 3** Anticipated parameters for organisms in the three IMTA systems tested in Eilat, Israel, based on 500 t feed per year

| |
|---|
| Seabream |
| FCR = 1.9; Feed protein content = 49% |
| Fish stocking density = 200 t $ha^{-1}$; Annual fish yield −300 t $ha^{-1}$ |
| Seabream farm gate price = €4 $kg^{-1}$ |
| Seaweed |
| Ammonia uptake rate –4 g $m^{-2}$ $day^{-1}$; ammonia uptake efficiency = 85% |
| Annual Ulva yield = 900 t $ha^{-1}$ |
| Seaweed (WW) price = €0.5 $kg^{-1}$ |
| Abalone |
| FCR = 12; stocking density = 25 kg $m^{-2}$ |
| Annual yield = 10 kg $m^{-2}$; |
| Farm gate price = €35 $kg^{-1}$ |
| Sea urchins |
| FCR = 8 t *Ulva* 1 t of production; stocking density = 10 kg $m^{-2}$ |
| Annual yield = 8 kg $m^{-2}$ |
| Farm gate price = €10 $kg^{-1}$ |
| Clams/Oysters |
| Clam annual yield = 6–8 kg $m^{-2}$ |
| Clams farm gate price = €4.5 $kg^{-1}$ |
| Oyster annual yield = 25 kg $m^{-3}$ |
| Oyster farm gate price = €3.5 $kg^{-1}$ |

### Biological Aspects

- To acquire the knowledge necessary to maintain the correct balance between nutrient production by the system's core organism, nutrient uptake capacity of microalgae and macroalgae, shellfish filtering efficiency, and macroalgivores' activity in the system
- To acquire the knowledge necessary to maintain steady populations of microalgae (mainly diatoms) for the filter feeders and of macroalgae for the macroalgivores within the system in order to avoid blooms and crashes
- To acquire the knowledge necessary for the efficient regeneration of the biodeposit (sludge) from the bottom back to dissolved nutrients for the macro- and microalgae
- To effectively control diseases of the cultured organisms in IMTA systems and transmission of pathogens between components of the system

### Engineering Aspects

- To reduce construction and operating costs by engineering improvements
- To minimize heat loss or gain in downstream components of the system
- To increase the use of greenhouse-covered modular systems, gravitation, low head upwelling, water semi-recirculation and other promising energy-saving methods

### Economical Aspects

- To render cost effective the use of the extensive areas required for cultivating micro- and macroalgae which cannot be done in a fully recirculating system and for which the facilities must thus be located not too far from the sea
- To develop and diversify the market of seaweed for human consumption from IMTA in Europe and North America
- To develop new markets and consumer acceptance of IMTA products

With the dramatic increase in population and food requirements, traditional extensive production systems cannot satisfy present and future market needs. Modern intensive monoculture systems are not ideal for mass production because they focus on few and expensive species, require high levels of resources, and produce undesirable wastes. To achieve high production rates and environmental conservation, food production using land-based IMTA systems is one of the most promising routes. The IMTA method assimilates expensive nitrogen waste into a valuable product that will increase profit for the farmer, improve FCR, diversify the mariculture products, create additional jobs, and, most importantly, reduce environmental pollution.

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# Marine Aquaculture in the Mediterranean

Dror L. Angel
Leon Recanati Institute for Maritime Studies, University of Haifa, Mt Carmel, Haifa, Israel

## Article Outline



## Glossary

**Bioassay (BIOlogical ASSAY)** A procedure to test the effect of a substance on living organisms, e.g., the effect of plant nutrients on plant growth rate.

**Chemotherapeutants** The use of chemicals to treat disease.

**Dead zones** Coastal areas that undergo seasonal hypoxia (low-oxygen), generally related to eutrophication events, whereafter many of the local (mainly benthic) animals die.

**Exotic species** An introduced or alien species living outside its natural range, which has been introduced by deliberate or accidental human activity.

**FCR (feed conversion ratio)** The efficiency at which an animal converts its food into biomass (body mass); FCR = mass of food eaten/increase in biomass.

**Immunostimulants** Chemicals used to stimulate the immune system by inducing activation or increasing activity of any of its components.

**Marine protected areas** Areas that restrict human activity (e.g., fishing, boating, coastal development) to protect living, nonliving, cultural, and/or historic resources.

**NIMBYism** "Not In My Back Yard"-ism; the practice of objecting to a human activity (generally commercial or industrial) that will take place near one's home.

**Oligotrophic** Waters that have low levels of nutrients and algae, high level of dissolved oxygen, and deep light penetration (i.e., clarity).

**Prebiotics** Food ingredients (e.g., soluble fiber) that stimulate the growth and/or activity of bacteria in the digestive system which are beneficial to the health of the body.

**Probiont** Living bacteria added to the environment and feed of reared animals and thought to benefit them by improving intestinal microbial balance, thereby inhibiting pathogenic bacteria.

**Protista** Unicellular (single-cell) eukaryotic organisms, e.g., foraminifera.

## Definition of the Subject

Fisheries and aquaculture play an important role in the economies of many countries; yet this fact is often overlooked as the focus, in many nations, is on provision of food primarily, if not exclusively, from terrestrial agriculture. The value of seafood products as a source of foreign currency is especially important in developing countries and in many cases may exceed the profits from certain agricultural products [1], though this fact also tends to evade common knowledge. The Mediterranean aquaculture sector continues to grow at a rate of close to 9% per year (since 1970) as compared to 3% per year for farmed meat production systems. If the growth of the aquaculture sector can be sustained, it is likely to fulfill the demand for aquatic food supplies by supplying >50% of the total aquatic food consumption within the next 5 years! Therefore, the emphasis here is on the review of the sustainable growth of a commercial activity within an enclosed sea with many conflicting multinational interests. Aquaculture includes the cultivation of finfish, shellfish, crustaceans, and algae; however, this review will focus primarily on Mediterranean finfish farming since many of the sustainability issues revolve around fish farms. There are many different facets (e.g., ecological, social, political, economic) to sustainable commercial activities and this review will touch on several, though not all, of the issues related to aquaculture and its sustainable development in the Mediterranean Sea region.

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## Introduction

### The Mediterranean Sea Environment

Although the term "environment" is often used to mean "ecology," the following description embraces the more holistic meaning, which includes the socio-economic aspects as well. The Mediterranean is a large, semi-enclosed sea bordered by 22 countries, with two distinct basins divided by a narrow, relatively shallow channel between Sicily in the north and Tunisia in the south. The areal division of the sea between the western and eastern basin is roughly 1/3:2/3. The eastern basin is somewhat more saline than the western basin, especially in the vicinity of the Suez Canal. The Mediterranean Sea has a wide range of seawater temperatures, from as low as 5°C in the Gulf of Trieste in the winter to 31°C off the coast of Libya in the summer [2]. The sea is oligotrophic and phosphorus limited [3] though some limited areas (such as parts of the northern Adriatic) may be eutrophic and it is warmer and more oligotrophic in its southern and eastern areas. Whereas the Mediterranean Sea accounts for only 1% of the world's ocean, it contains 6% of the world's marine species, including >400 endemic species of plants and animals [4]. Despite this impressive biodiversity, biomass is relatively low, mainly due to low primary production.

There are approximately 82 million people in the Mediterranean coastal zone: most in coastal cities and 32% of the population is in North Africa. Levels of development vary widely over the region. Tourism brings >100 million visitors to coastal areas annually, serving as a major source of seasonal population pressure and income and is thus a major competing sector with aquaculture. The Mediterranean Sea is a major shipping route, bridging between Europe and the Middle East and is a base for capture fisheries and mariculture. There are 75 marine protected areas (MPA) in the region, designed to protect unique and threatened resources and habitats such as the seagrass *Posidonia oceanica*, and breeding and nesting sites for endangered species, such as the loggerhead sea turtle (*Caretta caretta*). MPAs were also designated to encourage specific uses, such as sustainable tourism and regenerating fish stocks [5].

### A Brief History of Mediterranean Aquaculture

The earliest evidence of aquaculture activity in the Middle East is from the ancient Egyptians. An Egyptian frieze, dated from 2500 B.C., depicted men gathering fish from a pond in what may be the earliest record of such activities in this region [6, 7]. In the sixth and fifth centuries B.C., the Etruscans reared fish in marine farms and the Greeks grew mollusks [8]. Throughout the Roman empire, marine fish (mainly sea bass, sea bream, and mullets) and oysters were reared in special enclosures (e.g., piscines) along the coast [9–11], but this practice seems to have died out with the collapse of the empire and did not appear in the Mediterranean until the middle ages. It is not clear precisely when it began, but there are records of extensive aquaculture in lagoons in Italy, also known as valliculture, starting from around the fifteenth century. Europeans traditionally collected shellfish along the shores, but since the eighteenth century the French oyster industry added a more reliable source – shellfish reared in specialized gear in the intertidal zone. Shellfish aquaculture expanded in the nineteenth century and coastal cultivation spread throughout the Western Mediterranean and the northern Adriatic Sea.

In the second half of the twentieth century, aquaculture developed rapidly, mainly as a result of successful research into the life cycle of the farmed animals (reproduction and larval rearing), as well as physiology, nutrition, and engineering of farming systems [8].

### Main Forms of Mariculture (Culture Types and Species) in the Mediterranean

On a global scale, aquaculture production in the Mediterranean Sea is small, but not insignificant – especially with regard to the European demand for fresh seafood. Total aquaculture production in the Mediterranean Sea in 2006 was about 370,000 t [1] with 14% growth from 2000 to 2006, outpacing the growth of capture fisheries. It is noteworthy that the interannual variability in aquaculture production is lower than in capture fisheries (these have reached a plateau in terms of annual harvest), which may be a consideration of prime significance for business and decision-makers concerned with food security, coastal communities, and development.

Within the Mediterranean aquaculture sector, the most striking feature of production is the rate at which finfish have overtaken mussels as the dominant product. In 1990, finfish production accounted for less than 10,000 t as compared to approximately 90,000 t of mussels. In 2003, 180,000 t finfish and 150,000 t mussels were produced (49% and 40% of total production, respectively). Clam and oyster production were only 7% and 2%, respectively, and the remainder of production (∼2%) was crustaceans and seaweed. The main cultivated finfish species in the region are gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), and flathead gray mullet (*Mugil cephalus*). Greece, Turkey, Spain, and Italy were the four largest producers of sea bream and bass in 2006, comprising >90% of total Mediterranean production. Sea bream and bass are predominantly reared in net cages in coastal waters, whereas mullets are generally reared in ponds. The major producers of mullets are Egypt and Italy with Egypt generating more than 90% of global mullet production.

A fairly recent development is the farming of bluefin tuna in the Mediterranean, which mainly serves the Japanese sushi market. Tuna farming falls in between the definitions of a standard fishery, which is defined as "capture of wild stock" and aquaculture where fish are both bred and reared in captivity. Because tuna farming is a "postharvest" practice, it is not governed by the regulations of GFCM or ICCAT [12] and as a result there was unregulated growth in this sector, putting heavy pressure on the endangered Mediterranean wild stocks. Concerted efforts are being made to create brood stocks and hatcheries to enable the cultivation of bluefin tuna by the traditional aquaculture methods to release pressure on the endangered Mediterranean wild stocks.

## Sustainable Marine Aquaculture in the Mediterranean

One of the features of marine aquaculture in the Mediterranean is that it is developing rapidly in response to a large and ever-growing demand for seafood. This demand was traditionally supplied by fisheries, but the drop in landings in recent decades as a result of overfishing has opened the path for sustainable alternatives to provision of seafood, namely aquaculture. That said, mariculture needs to operate in a manner that will minimize negative impacts on the marine environment, on wild stocks, and on other uses of the seas. Thus, sustainable aquaculture must ensure "*economic viability, social equity and acceptable environmental impacts*" [13].

It is obvious that aquaculture activity must be profitable to succeed, but there are many criteria to profitability and *economic viability* and these may vary considerably in countries that are at different stages of economic development (the process whereby an economic activity develops the technology and experience needed to operate successfully) or that have different interests in mind. In some developing countries, aquaculture may serve as a much needed food and protein source for local consumption, whereas other developing countries may prefer to export their aquaculture production for economic benefit.

Another component of sustainability is *social equity*. Societal equity depends on cultural norms and tendencies of society and varies considerably among the Mediterranean countries. It is probably the most difficult aspect of sustainability to consider because of its intrinsic variability.

*Environmental* "*acceptability*" is also a difficult issue because of the obvious question: "acceptable by whom?" In order to address this, one needs to consider where the aquaculture activity takes place, who are the stakeholders and how this activity may be conducted in such a manner that it will be acceptable by as many stakeholders as possible. The first aspect of sustainability, discussed below, is the public perception of aquaculture since public opinion may play an important role in the success or failure of the industry. In addition to the various social ramifications, "environmental acceptability" includes the effects of aquaculture on its surroundings and on the ecosystem. The following sections list several of the environmental issues that affect or are affected by Mediterranean aquaculture and a discussion of what is being done about them to enhance the sustainability of this sector.

### Public Perception of Aquaculture

The *image* of fish farming varies considerably among different countries and can have a strong effect on the

sustainability of the industry. In some northern European countries, the public considers aquaculture in a positive light as a means to enhance food safety and security. In comparison, many southern European countries have a generally negative attitude toward farmed fish as these are considered inferior in taste and health value in comparison to wild-caught ("natural") fish [14, 15]. Numerous negative connotations are associated with marine aquaculture, including: "pollution causing eutrophication," "discharge of antibiotics and harmful chemicals into the environment," "genetic dilution/pollution of wild fish stocks," and "negative visual impact on the coasts."

The public perception is very important for both producers and coastal zone managers since there are many factors that are stacked against the aquaculture sector [16, 17]. These include lack of knowledge on many aspects of the coastal environment, the weakness of a small industry, competition with tourism and other coastal stakeholders, and increasing political power of local environmental lobbies and associations. These lead to non-sustainable situations, including loss of licenses, leases and markets, and reduced diversity in the coastal economy.

The social acceptability of aquaculture was examined at two Greek islands [18] and revealed that residents were more likely to be opposed to aquaculture if they thought that the fish farms would pollute the environment. A study conducted in Israel [19] evaluated public attitudes toward aquaculture and concluded that although most citizens were not terribly well informed in the implications of aquaculture on tourism and environmental issues, the majority are in favor of marine aquaculture. It is noteworthy that this lack of familiarity with aquaculture and aquaculture implications was also observed among the public surveyed in such countries as Scotland [20], Australia [16], and Germany (Schultz, unpublished).

Although the above focuses on the attitudes of the lay public toward aquaculture, it is possible that the opinion of stakeholders is equally (or more) important, despite the fact that the number of stakeholders is usually smaller. Competition over the coastal zone is one of the major sustainability issues that Mediterranean aquaculture faces on a regular and large-scale basis. The competition is especially severe between aquaculture and tourism since the Mediterranean attracts about 30% of the volume of global tourism annually and this is expected to increase over time. There are many examples of such competition, and one of the more recent clashes between the tourism and aquaculture sectors occurred in Turkey in 2008–2009, resulting in a major shift in legislation and in aquaculture lease requirements.

**Measures to Improve the Public Attitudes Toward Aquaculture** The negative attitudes toward aquaculture are largely a result of ignorance. The media often presents NGO views and opinions in their description of the fish-farming industry, and many of the facts presented are incorrect. The way to correct some of the misconceptions surrounding aquaculture is by preparing a well-planned outreach and educational program geared to reach as many households as possible. There are myths and misconceptions regarding such things as how fish are reared and the densities at which they are stocked, the safety of the feed used, the quality and healthiness of farmed versus wild fish, etc. Preparation of an aquaculture "module" to be taught at schools is an effective way to reach and educate future stakeholders and decision-makers. Another measure that could reduce conflict between aquaculture and other coastal stakeholders is a search for synergies among the stakeholders that would enable multiple use of the coastal zone [21]. Promotion of organic and other types of certification programs to increase public confidence in aquaculture practices and products would also improve public attitude toward this sector.

## Benthic Impacts

In the 1990s, the study of the interactions of Mediterranean marine aquaculture with the environment focused on the negative impacts of the industry since most of the early research on salmon farms documented heavy benthic loading, which caused serious damage to underlying seafloor communities and in some cases to the water column as well [22–26]. Benthic organic enrichment that often occurs under intensive finfish farms rapidly leads to hypoxia and anoxia in the sediments. Anoxic sediments support bacterial sulfate reduction, generally leading to an increase in sediment hydrogen sulfide [27]; conditions that are

noxious, at best and often lethal to macro- and meiofauna [28]. Although abundances of macrofauna in Mediterranean sediments are considerably lower than the abundances found in temperate regions [29–31], defaunation under fish farms strongly reduces benthic bioturbation (i.e., aeration of the sediments) and leads to accumulation of reduced compounds and organic matter therein. If the farm is situated at a site with limited flushing and circulation, the depth and aerial extent of the impacted sediments may grow with time, creating localized "dead zones." Moreover, when methane accumulates in and bubbles out of anoxic sediments, noxious chemicals such as ammonia and hydrogen sulfide may affect the cultivated fish in the overlying cages.

Because the Mediterranean Sea is largely oligotrophic, and fish farming is generally not practiced at sites with poor flushing, the phenomena described above are not common. At a few sites with limited water circulation, for example, some farms in Croatia and Greece, organic enrichment of the seafloor and local impacts were observed, but these were exceptional and sediment conditions under Mediterranean fish farms are generally less impacted.

At those sites that showed evidence of impacted sediments, the visible effects generally did not extend beyond tens of meters from the edge of the perimeter of the farm [32], though the situation at each farm is different as a result of site-specific currents, depth, bathymetry, etc. The determination of the extent of impacted sediments and benthos (distance from the farm) is subjective and may be strongly affected by the method used. Organic matter determinations, visual inspection, and macrofauna indices are often the methods used to assess the state of the sediments and these clearly show a local effect that diminishes with increasing distance from the point source. However, more sophisticated analyses involving stable isotope signatures of farm effluents indicate that the aquaculture effluents may be detected as far away as 1–2 km from the farms [33–35]. It is very important to qualify the meaning of these measurements because they may be used to make a point about the extent of fish farm effects, but the real issue at hand is the extent of "significant impact." The distribution of small suspended particles over great distances will only constitute a significant impact if the flux of these particles is large and in the case of Mediterranean fish farms, the flux of very small suspended particles is small [36]. Therefore – it is essential to emphasize the difference between qualitative and quantitative effects.

**Measures to Reduce Benthic Impacts** Despite the fact that benthic loading is generally not a major issue in the Mediterranean, a number of different approaches are employed to increase feeding efficiency and reduce benthic loading. Feeding efficiency is not only an environmental issue, but also a major economic consideration since one of the greatest cost factors in intensive fish farming is the formulated feed. Feeding efficiency includes optimizing the composition of the feed (optimal digestibility) to maximize growth and minimize waste at the lowest possible cost, as well as feed delivery. Considerable efforts are invested by feed companies and fish nutritionists to optimize feed for the various strains of cultivated Mediterranean finfish [37, 38] and during recent years, sea bream and sea bass feed conversion ratios (FCR) have been substantially improved, largely (though not exclusively) due to improved diets and feed delivery. Feed delivery includes the optimal feeding regime whereby feed is provided to the caged fish in suitable portions and at the correct intervals to both maximize growth and health and minimize loss to the surrounding waters. Low-tech feeding involves delivery of pelleted feed to fish either manually by hand, or with the aid of a compressor and regulating the amount according to the response of the fish. High-tech systems include feeding programs that are computerized and customized to each individual cage to optimize delivery of feed to the stock. Another sophistication is the use of submerged Doppler systems (e.g., Doppler Pellet Sensor) that detect when fish stop feeding (increase in the flux of pellets to the bottom of the cages), and send signals to cause the automated feeders to cease feeding (http://www.akvagroup.com/). Many of the above are technologies that were developed outside of the Mediterranean, but as they are also applicable to sea bream and bass production, they are widely used by this sector. One of the more recent developments in Mediterranean aquaculture was the tuna-fattening process, which offered large profits to the farmers. Although it is arguable whether this process should actually be

qualified as aquaculture, the environmental ramifications were clear. The penned fish are fed freshly caught or frozen fish rather than pelleted feed and release large amounts of waste (greater than would be released from pelleted food) to the seafloor and have rather high feed conversion ratios (FCRs) 10:1–20:1 as compared to the FCR of sea bream (2:1) or salmon (1:1). Research is currently ongoing to develop artificial diets to create a better FCR for the tuna and to reduce the reliance and fishing pressure on small pelagic fish (e.g., [39]).

## Water Quality

The sustainability of any human activity is a function of the nature of the receiving or host environment and in the case of aquaculture this is the basis for estimating the assimilative, holding, or carrying capacity [40]. At a few sites with restricted water exchange, for example, lagoons, there were reports of eutrophication problems [41–43] as the loading of organic and inorganic nutrients clearly exceeded the capacity of the environment to assimilate these [44]. Sites where such self-pollution problems emerge suggest that the preliminary environmental impact assessment and site selection procedures were not carried out properly. In the oligotrophic waters of the eastern Mediterranean, there are generally no reports of eutrophication or degraded water quality related to finfish or shellfish farms [45–47] and this was interpreted as the ability of the oligotrophic system to successfully assimilate the nutrients released by the farms. In an effort to understand whether nutrient release from aquaculture might have large-scale effects on the Mediterranean ecosystem, Karakassis et al. [48] employed a model to examine various production scenarios. They concluded that if aquaculture continues to grow and expand at present rates, farm wastes may increase overall nutrient (mainly N and P) levels by 1%, however, this is a general assessment and does not take into account localized effects. As suggested by Pitta et al. [49] in a study of three different Greek farms, it is likely that dispersion and dilution of the nutrients, combined with efficient herbivore grazing of algae (that develop from the released nutrients) were the reason for the absence of eutrophication around fish farms.

Although water quality is generally not affected, fish farms that operated over or near seagrass beds (especially *Posidonia oceanica*) exerted a clear effect on these [50, 51] and it was proposed that this may be related to the enhanced flux of dissolved and particulate nutrients from aquaculture. In an attempt to identify the effect of the plume of nutrients released from fish farms on water quality, Dalsgaard et al. [52] devised an innovative "bioassay" to measure the effect of dissolved nutrients released from fish farms on micro- and macro-algal production. They determined that primary productivity decreased with distance from the fish farms, yet by comparing bioassays with and without grazer exclusion, Pitta et al. [53] found that planktonic grazers (probably protista) play a key role in transferring nutrients up the food web.

**Measures to Reduce Effects on the Water Column**

One of the primary considerations when evaluating the suitability of sites for aquaculture is how they will interact with the surrounding marine system [54]. It does not pay, for example, to place net cage farms in shallow, poorly flushed waters (e.g., lagoons) because the organic and inorganic enrichment may affect both the marine ecosystem and the farmed organisms. Nevertheless, some farms have been deployed in unsuitable locations and these need to be relocated to allow the environment to recover and to enable the healthy growth of farmed finfish.

One of the early water quality problems associated with Mediterranean fish farms was the presence of an oily film around the cages. This was generally related to the large percentage of dust (pulverized feed pellets) in the pelleted food, which is not available to the farmed fish. Because this causes considerable loss to the farmers, and reduced water quality (stimulated bacterial growth also depletes the water of essential dissolved oxygen), the problem was rapidly addressed and most of the pelleted feeds are now extruded to improve pellet integrity and reduce feed loss and feed dust is collected and recycled.

A similar problem was identified in the tuna-penning industry. Unlike sea bream and bass that feed on formulated pellets, tuna are fed whole (preferably oily) fish such as sardine, anchovy, and mackerel. When these fish are offered to the tuna, the water around the pens often has an oily film and emits a strong smell. Moreover, in some cases, divers have complained of poor visibility near the pens. As described above,

research is ongoing to develop artificial diets for tuna [55] that will address not only the problems related to feeding with fresh fish but also the water quality problems.

## Disease

Intensive aquaculture systems are very susceptible to disasters such as loss of the farmed stock. Among the various causes of such disasters, disease outbreaks rank highest [56] and may lead to great losses within a very short period of time.

Most finfish cage farms in the Mediterranean are intensive, that is, they have high stocking density in order to be economically profitable and to compensate for the low profit margin of sea bream and sea bass, the main species reared in this region. Although cage stocking densities are usually $<25$ kg $m^{-3}$, in some farms stocking densities are higher and such conditions may cause a reduction in fish growth rates, suppression of immune mechanisms [57–59], and ultimately greater susceptibly to disease agents, including opportunistic bacterial and viral pathogens and eukaryotic parasites [60]. Current estimates of average mortalities for farmed sea bream and sea bass as a result of disease are 10% and 20%, respectively, for growth from juvenile to market size (350 g) fish. In many cases, the profit margin for these fish is not much higher than 10–20%, which has therefore obliged the aquaculture sector to consider various options to address this problem. Moreover, there is concern regarding the potential transmission of disease from the farmed stock to wild fish, based on studies of disease transfer among Atlantic salmon (e.g., [61]). It is noteworthy that although there are numerous examples of disease exchange between caged and wild fish (e.g., [62–64]) in the Mediterranean, and other seas, most of these are not clearly understood [65, 66] and their effect on native stocks is unclear.

**Measures to Reduce Disease Outbreaks** Numerous antibiotics have been tested against the common farmed fish diseases and there are currently treatments available for most bacterial fish pathogens [67]. However, the routine use of antibiotics in marine aquaculture is problematic and has declined for a number of reasons. First, as specified above, there are concerns related to human and environmental health and safety. Second, although many of these drugs work well in freshwater, some of the major antibiotics, such as quinolones and tetracyclines interact with the divalent cations that are abundant in seawater (mostly $Mg^{2+}$ and $Ca^{2+}$) which massively reduces their function and efficacy [68, 69]. Moreover, there is no "harmonization" regarding antibiotics use among Mediterranean countries and the list of pharmaceuticals licensed for fish varies from country to country, complicating international trade and marketing.

In addition to bacterial pathogens, there are several parasitic diseases that may stunt growth rates, cause loss of fecundity, and even mortality in Mediterranean fish. These include various protozoa and metazoa, which are classified as ecto- and endoparasites according to their distribution on/in the fish. Pathologists consider the myxosporeans *Myxidium leei*, *Polysporoplasma sparis*, and *Ceratomyxa* sp., isopods, copepods, and monogenean infections among the more problematic parasites.

Athanassopoulou et al. [70] reviewed the drugs used against a variety of parasites and found that amprolium and sanilomycin were the most effective against myxosporans in cultivated breams. Moreover, extracts from oregano revealed anti-myxosporan as well as antibacterial properties. Ivermectin and deltamethrin – drugs used to combat sea lice, have also been tested against copepod and isopod infections in sea bass and were fairly effective, but they tend to become toxic to the fish at fairly low levels.

In order to limit the use of antibiotics and other chemotherapeutants, the European Union established the "Maximum Residue Limit" (MRL) regulation, which monitors the presence of these drugs in all agriculture and aquaculture products and this has had a dramatic effect on the use of therapeutants. Because the MRL differs among countries insofar as which compounds are regulated and which are not, there is a lot of work ahead, but despite this, the trend looks very promising.

Vaccines are one of the preferred measures for prevention of disease outbreaks, however because the Mediterranean finfish market is still fairly small, only a limited number of vaccines have been developed for commercial use. Moreover, consumer concerns and increasing restrictions regarding their use have led the

industry to consider other alternatives to disease "management" [71, 72].

There are other alternatives to the use of chemotherapeutants and vaccines against disease. One of the key factors in the prevention of disease is good husbandry, which focuses on minimizing stress to the farmed stock. This includes proper stocking densities, optimal nutrition, sanitary practices, use of vaccines, and probiotics [66, 73]. The practice of good husbandry ensures fish are healthy and able to resist various disease agents naturally found in their environment. When they become stressed, the dietary requirements of fish for nutrients and vitamins change and a diet that compensates for such needs may optimize the growth of fish in captivity.

In recent years, it has become clear that the integrity of the gastrointestinal tract is essential in defense against pathogen attack as well as in proper endocrine and osmoregulatory activity. In recognition of this, Dimitroglou et al. [74] added the mannan oligosaccharide, Bio-Mos® (Alltech Inc, USA) to the diet of several marine fish including gilthead sea bream and found that this improved the gut morphology.

It is assumed that one of the roles that the mannan plays in protection of the fish is agglutination of pathogenic bacteria, which prevents their colonization of the gut. Indeed, the application of Bio-Mos significantly reduced the bacterial load in fish guts by reducing the biomass of aerobically cultivated bacteria [74]. Torrecillas et al. [75, 76] applied Bio-Mos to sea bass juvenile diets and found that it improved growth rates by 10%. Moreover, challenge trials using *Vibrio alginolyticus* showed that Bio-Mos fed sea bass had fewer of the pathogenic vibrio in their gut.

In recognition of the essential role of healthy gut flora in fish, especially in young fish, the use of immunostimulants, prebiotic, and/or probiotic bacteria have been proposed as a means to reduce gut colonization by pathogens [77], thereby improving the survival of cultured fish. Probiotics involves the addition of nonpathogenic bacteria to the diet and water of fish with the aim of loading the gut with bacteria that will prevent colonization by competing pathogens. The use of prebiotics and immunostimulants focuses on boosting the fish immune system so that the fish may more readily recognize and repel pathogen gut colonization. Although research has been conducted on the use of probiotics in Mediterranean aquaculture, (e.g., [78–80]), this approach has not successfully replaced the use of antibiotics to combat disease. One of the problems related to the use of probiotic bacteria is concern that these may not be as safe as they are supposed to be and their use may lead to other problems rather than a sustainable solution in the battle against disease.

Immunostimulants are commonly used in finfish farming to reduce the risk of disease by stimulating the protective activity of the immune system. The common forms of immunostimulants used in sea bream and sea bass aquaculture include ascorbic acid, a-tocopherol, and glucans [81, 82], which are added to the feed. Their presence appears to enhance antibacterial lysozyme activity and other indicators of disease resistance, but there is considerable discussion about their effectiveness due to the inherently wide range in concentrations and activities of the disease resistance molecules in fish serum.

Another approach to reduce the risk of disease is by means of classical selection/breeding for disease resistance by means of selective breeding programs [83]. The understanding of immune regulatory genes responsible for resistance to finfish pathogens is still in its infancy in Mediterranean aquaculture, but this field is rapidly expanding and it is anticipated that genetically superior lines will dominate the populations of fish reared in intensive aquaculture [84].

### Escapes

In addition to problems related to disease and fluctuating profitability of aquaculture operations, fish farmers are also concerned with keeping their fish within the cages so that these can be marketed at the end of the growth cycle. There are many factors that may lead to loss of the farmed stock, including storms that may physically damage the net cages, predators (e.g., sharks, dolphins, bluefish, seals) that may bite the nets in their attempt to eat the enclosed fish, human error (e.g., during replacement of net cages or during harvest), poachers that cut the nets to catch fish, collision of ships with cage farms, etc. All of these generally result in the release of farmed fish to the surrounding environment, involving financial loss to the farmer and potential environmental problems related to genetic and ecological interactions of the escapees with the wild fish. At present, there are an

estimated >1 billion fish; mostly sea bream and sea bass in net cage farms throughout the Mediterranean as compared to much smaller stocks of the wild populations of these species [85], so the potential impact of escapees is considerable. Because many Mediterranean countries do not require farmers to report escapes, there are no reliable data on the frequency of escapes, however, it is assumed that the percentages of escapees are similar to those reported in Norway [86], ranging between 0% and 6%. In addition to genetic "pollution" of the wild-stock gene pool, and potential competition between escapees and wild fish over the same habitat and food resources, there is also concern regarding the spread of disease from farmed fish to wild fish populations [87].

**Measures to Reduce Escapes and Damage due to Escapes** As the volume of aquaculture production increases in the Mediterranean to match demand, and with the anticipated addition of North Africa to the fish-producing countries, there is a growing need for regulation in order to minimize problems related to escapes. In order to appreciate the scale of escapes from Mediterranean aquaculture, there is a need to legislate reporting of escape events, as is currently done in other parts of the world. Moreover, several new finfish species have been domesticated and their potential effect, as escapees, on wild populations and on the ecosystem need to be assessed. In addition, in order to assess escape impacts, it is useful to be able to track the escaped fish, as described by Triantaphyllidis [88].

There are many measures that may be employed to reduce the risk of escapes from fish cages. Storm damage to farm systems is one of the major causes of escapes and employment of a reliable standard, as practiced in Norway (NS 9415 – requirements for design and operation of marine fish farms) is a promising approach to reduce such risks. Even sturdy, reliable cages are occasionally damaged by especially strong storms, but most of the surface wave energy is concentrated in the upper 10 or 15 m of the water column [85]. Submersible cage systems designed for open sea conditions, such as the Sub-flex system (www.subflex.org) and the Ocean-Spar system (www.oceanspar.com/) are an option to reduce mechanical stress to net cages in high-energy environments. Added advantages of submersible cage systems include the reduced risk of collisions with maritime vessels and the reduced visibility following the "out of sight–out of mind" solution to NIMBYism. Human poachers are a problem that may be reduced by vigilance and by cooperation with the local police or security forces. Marine predators that bite net cages from the outside may be deterred by using stronger materials, though this has financial consequences, or by embedding chemical deterrents in the net material. Several farmed species tend to bite the net material from the inside and this may create holes enabling escapes. The biting may be prevented by using taste deterrents, as described for predators, or stronger material that will be more bite resistant. Moe et al. [86] suggest making the cage environment more "appealing" or stimulating to reduce gnawing on the net mesh which they attribute to boredom.

In addition to reducing the risk and frequency of escapes, there is also a need to reduce the impacts caused by the escaped fish. One direction that is being tested is the development of sterile triploid sea bream and sea bass that will not be able to pass on their genes to wild fish. Another possibility is the recapture of the escaped fish, but this direction is still in early developmental stages. The location of fish farms relative to areas of high ecological sensitivity or to spawning grounds should be one of the major considerations in light of the possibility that some of the stocked fish may escape.

## Introduced Exotic Species

Invasive species are probably the cause of the greatest ecological problems identified over the past century, not only in terrestrial but also in aquatic and marine systems [4]. This problem has intensified over the past 20–30 years, as the volume of intercontinental traffic has increased. Aquatic invasive species are a major threat to marine biodiversity and impact human health and the economy [89]. There are numerous examples of the impacts of invasives on human welfare and environmental health, for example, the invasion of the Black Sea by the exotic ctenophore *Mnemiopsis leydi*, which caused the collapse of most of the local fisheries [90]; invasion of the eastern Mediterranean by the Red Sea medusa, *Rhopilema nomadica*, which has

heavily impacted Israeli and Turkish fisheries, tourism and coastal facilities [91].

In the eastern Mediterranean, exotic introductions are mainly channeled through the Suez Canal whereas most of the successful invaders in the western Mediterranean have been introduced by ships and via aquaculture [92].

Species introductions via aquaculture activities may be intentional or accidental, though the consequences are generally similar. Intentional introductions generally include the import of an exotic species and its release into the environment, without the intention that it spreads and dominates its new habitat. Examples include shellfish such as the Japanese oyster that was brought to France and spread rapidly throughout French coastal waters and certain species of sport fish that were intentionally released in northwestern US waters. The majority of introductions are not intentional but rather accidental and may occur in a number of ways. One common example of an accidental introduction is the transfer of a local species of oyster from a hatchery to the coast in a restocking program and the accidental release of an associated seaweed with the oysters. In another case, recreational boaters did not thoroughly wash the bottom of their boat after a holiday in a given bay and when they transported the boat back to their own shore, they brought with them a cryptic gastropod which subsequently invaded the new environment and decimated the local clam population.

**Measures to Reduce the Invasion of Exotic Aquatic Species and Associated Damages** In order to avoid the various risks involved in the use of exotic species, it is essential to rear/grow native species, as a rule. In many cases, the commercially attractive species are not native and farmers prefer to culture nonnative species. Introduced species may only be considered after taking all required precautions as specified in the ICES *Code of Practices on the Introductions and Transfers of Marine Organisms* [93] and the report on *Alien Species in Aquaculture* by Hewitt et al. [94]. Because the introduced species may escape and invade either local or neighboring environments, with implications for marine biodiversity, there is a need for both regional and international collaboration to address transboundary introductions and invasion issues, as discussed in UNEP [92].

## The Mediterranean Aquaculture Market

The dominant species currently reared in the Mediterranean Sea are sea bream and sea bass [95]. These are native species that have been traditionally fished and eaten for centuries in many of the Mediterranean countries. Aquaculture has greatly increased the availability of these fish to the public and as production has increased, the price of the farmed fish has dropped dramatically so that in many cases its profitability is questionable. One of the important elements of a sector's sustainability is its economic performance yet the current trend in the Mediterranean is a plateau in profitability, that is, stagnation due to a glut in production of the two main species and a concurrent drop in their market value.

**Alternative Aquaculture Species** In order to survive and grow, the Mediterranean aquaculture sector needs to diversify its marine finfish production and include species with high market value. There are many native Mediterranean species that have a market because they are caught and sold by fishers and are suitable for cage culture. These include several species that have already been successfully reared in the eastern Mediterranean, such as Grey mullet (*Mugil cephalus*), Dover sole (*Solea solea*), Meagre (*Argyrosomus regius*), Sharp snout sea bream (*Diplodus puntazzo*), White bream (*Diplodus sargus*), Red porgy (*Pagrus pagrus*), Shi drum (*Umbrina Cirossa*), Striped sea bream (*Lithognathus mormyrus*), Pandora (*Pagellus erythrinus*). Although these fish are commercially available for aquaculture, there are several bottlenecks that prevent large-scale production. These include lack of knowledge regarding their nutritional requirements, lack of farm facilities for production, slow growth rates (may be related to nutrition or other problems), sensitivity to certain pathogens.

## Ecosystem Effects

It has been shown that Mediterranean fish farms generally have a local effect, primarily on the underlying benthos, as described above, yet within a short distance from the cages, this effect rapidly dissipates. It has been suggested that the large load of nutrients that pass via the farmed fish into the marine environment are rapidly processed by the biota, yet may exert some

ecosystem effects. This hypothesis was tested by comparing the biological/chemical composition of seawater from fish-farming zones (within 2–3 nautical miles of fish farms) versus nonfarm zones (20 nautical miles of fish farms) in three parts of the Aegean sea in May and in September [49]. The data indicate that there is rapid transfer of nutrients up the food web, from the primary producers, via herbivores [53] to fish [96, 97]. These findings may be interpreted in a number of ways and their ramifications are debatable. If the precautionary approach is adopted, it is not clear what sort of implications these ecosystem-level changes may have and so they should be regarded with caution. On the other hand, if fish farms increase the size of natural fisheries, providing fishermen with an increased catch, this may be regarded as a positive externality of aquaculture (positive socioeconomic impact), which should be encouraged.

### Seagrasses

One of the unique features in the Mediterranean Sea is the seagrass meadows of *Posidonia oceanica*. This slow-growing seagrass species occurs exclusively in the Mediterranean and grows best in clear, oligotrophic waters [98]. *P. oceanica* provides many ecosystem services, such as seabed stabilization, provision of a complex habitat to many larval and juvenile animals, oxygen production/release and long-term storage of $CO_2$ as plant tissue. Due to their slow growth rates, there is concern that these seagrass beds will not manage to recover if damaged and this important ecosystem and the services it provides may be lost. Marine botanists have calculated that some clonal colonies of *P. oceanica* may be 100,000 years old, that is, these are probably the largest and oldest-known living "organisms" on earth (http://en.wikipedia.org/wiki/Posidonia_oceanica). Because of their unique features, important ecological role and relatively low resilience to damage there is a strong movement in many Mediterranean countries to conserve and protect seagrass meadows from pollution, coastal development, trawling, and aquaculture. Recent work indicates that *P. oceanica* meadows located near or under fish farms have sustained considerable loss, including reduced meadow density, high shoot mortality rates (50-Diaz-Almela et al. 2008), increased epiphyte cover [99, 100] and very slow recovery rates following farm removal [101]. An analysis of several variables that may cause the observed damage to *P. oceanica*, in the context of the *MedVeg* project, has identified the deposition of particulate organic matter from the farms onto the seagrasses as the main factor leading to seagrass decline [102].

**Measures to Protect Seagrass Meadows** A set of recommendations were published by Pergent-Martini et al. [103] for the protection of Posidonia from fish farms, guided by the precautionary principle. These specified that: (a) Fish farms should not be situated directly over *P. oceanica* and *Cymodocea nodosa* (another important seagrass) meadows. (b) If seagrasses grow where a farm is planned, cages should be located at least 200 m from the nearest meadow. (c) Because these seagrasses generally occur at depths shallower than 45 m, farms should be set up at depths of 45–50 m where possible. (d) Environmental Impact Studies that relate to all seagrasses in the region should precede all lease requests to set up a fish farm. (e) If there are *P. oceanica* meadows near fish farms, these should be examined every 4 years to assure they have not been affected by the farming activity. On the basis of more recent findings, Holmer et al. [102] recommended to increase the distance between seagrass beds and fish farms to 400 m and to establish permanent seagrass plots to enable annual monitoring and sampling for seagrass health.

## Future Directions

In the early 1990s, finfish aquaculture was generally a novelty in most parts of the Mediterranean, but this has changed radically during the past 20 years, as cage culture has spread throughout the region. Aquaculture is one of the fastest growing sectors worldwide and in the Mediterranean and it has many advantages over other food production industries, but in order to maintain a "green" image, aquaculture production and development must be sustainable. Progress has been made in many aspects of aquaculture technology but there are several areas that require attention and improvements in order to make this industry more environmentally and socioeconomically sustainable. Although numerous projects have focused on understanding the environmental interactions of aquaculture, the calculation of a reliable "carrying capacity" for aquaculture in a given water body is still generally

beyond our means, that is, there is a need for further study of ecological processes on a variety of different scales with respect to fish farms. Because there are so many different types of habitats and ecosystems within the Mediterranean Sea (e.g., hard vs. soft seafloors, Adriatic vs. Levant, etc.), it is essential that the ecological and socioeconomic research address region-specific issues [45].

As aquaculture expands into new areas and new species, there is added urgency to improve the understanding of fish pathology in Mediterranean systems. In addition to bacterial diseases, there is a need for research into antihelminthic treatments, and better understanding of life cycles and early diagnostics for many of the Mediterranean parasites. In view of EU policies concerning reduction of chemical use in the aquatic environment, the prudent and effective use of chemotherapeutants is essential. This may be achieved by combining therapeutic treatments with such health management strategies as breeding of tolerant fish, improving water quality, and vaccination.

Escaped fish may impact wild fish through competition, predation, habitat displacement, gene pool dilution, etc. In an attempt to reduce the numbers of escapees, progress is being made (e.g., in the EU project "Prevent Escape," which includes several partners from Mediterranean countries) in the design of cages that should be more damage resistant and in devising strategies to track the escapees and to reduce migration away from the breeched cages.

### A Need for Legislation

One of the areas that urgently requires attention to enable development of the sector is legislation since this aspect is inadequately addressed in many Mediterranean countries. Moreover, in many countries that are active in aquaculture, there is a policy vacuum with regard to this sector. There is a need for clear rules and standards for licensing, planning, environmental impact assessment (EIA), administrative organization, and coordination. In the absence of clarity and transparency in such matters, investors and entrepreneurs will not take the risks involved in establishing aquaculture operations and the development of the industry will be retarded and sluggish. In a review of the legal obstacles to aquaculture, Van Houtte [104] included: (a) the legal status of water used (public or privately owned), the nature of water used (marine, brackish, or freshwater); (b) the legal status and nature of the land used (coastal vs. inland; private vs. public); and (c) the need for government regulation of aquaculture, and related activities. Moreover, the lack of coordination among public and regulatory agencies with regard to the EIA process, planning, etc. complicates the aquaculture application process. To further complicate matters, the permit application process is complex, cumbersome and very time consuming. The number of laws, regulations, rules, and procedures involved in the application process is large and many different authorities are involved at several levels. On top of that, the application requirements vary widely from country to country and in some countries, aquaculture legislation may vary internally on a provincial or regional basis.

One of the most problematic policy issues has to do with site selection and site allocation for aquaculture. As an economic activity that takes place, and has an effect on the littoral, aquaculture competes with many other uses of the coastal zone and needs to be included in Mediterranean coastal planning and management schemes. In recognition of the rapidly growing sector, in 2002 the European Union acknowledged that planning and coastal management would be among the major challenges facing European aquaculture. This was reinforced by the recent EU [105] communication, which emphasizes that "area choice is crucial and spatial planning has a key role to play in providing guidance and reliable data for the location of an economic activity, giving certainty to investors, avoiding conflicts and finding synergies between activities and environments with the ultimate aim of sustainable development" and invites all Member States to "develop marine spatial planning systems, in which they fully recognize the strategic importance of aquaculture."

One of the options chosen by some Mediterranean countries is zoning, that is, allocating a specific area for aquaculture as a means to reduce conflicts between coastal activities. In principle, this sort of approach simplifies things, provided: (a) the criteria used for selection of the aquaculture zones were appropriate and (b) the decisions regarding zoning involved the stakeholders and their interests. It is noteworthy that although there is aquaculture zoning in some

countries, aquaculture jurisdiction generally falls under regional governance, that is, there are no national zoning plans in the Mediterranean [54]. Although zoning is probably one of the better options for site selection, the lack of national coordination regarding the allocation of space for aquaculture will probably increase conflicts with time, thereby jeapordizing the sustainability of the industry. It would therefore be prudent to promote national zoning policy for aquaculture in the Mediterranean.

The conflict over space is fierce in the coastal zone as there are many competing stakeholders and one of the solutions to this is to go offshore [95, 106]. There have been many initiatives over the past few decades promoting offshore or open-ocean aquaculture, including several international conferences in the Mediterranean; however, a number of obstacles have prevented the realization of this concept. These obstacles include (a) economic feasibility of such ventures; (b) engineering and technological solutions for aquaculture in sites exposed to oceanic conditions; (c) international and national (government) support for an offshore aquaculture industry; (d) investors willing to take the risks involved in offshore aquaculture; (e) lack of understanding of the ecological ramifications (water column and benthos; local and regional effects) of large-scale aquaculture in exposed sites; and (f) the biological effects of cultivation in exposed conditions (storms, currents, predators, etc.) on the farmed stock, and other similar issues. At present, there are a few Mediterranean fish farms situated in exposed, offshore sites, but these are the exception rather than the rule, and most farms are situated in protected or semi-sheltered sites. A move away from the coastal zone into offshore waters will probably become a reality rather than an option in the near future and the aquaculture sector stands to benefit if it can accept this and help establish the scientific basis and technology in advance.

### Integrated Aquaculture

Another option that makes considerable ecological and economic sense is an integration of different forms of aquaculture within the same farm. By arranging systems for rearing finfish (a form of "fed" aquaculture) adjacent to systems for growing shellfish and/or seaweeds (extractive aquaculture), it may be possible to increase farm sustainability on a number of levels. On the ecological level, shellfish and algae are called "extractive" because they extract their nutrients or food from within the system (autochthonous), and can therefore help reduce the nutrient loads from fish farms. Finfish are usually "fed" with feed that is manufactured from materials that come from outside the system (allochthonous) and the release of wastes and uneaten feed from the farms may affect water and sediment quality and even cause eutrophication. On the social level, cultivation of different products as compared to monoculture will require greater manpower and expertise and create the opportunity for greater employment, both within the farms and in the form of support services. On the economic level, additional crops should increase farm profitability, provided the filtering organisms are able to absorb the nutrients efficiently and they fetch a good price at market. Moreover, by diversifying the cultured stock, the farmer protects himself from risks related to market fluctuations, storms, and disease. Integrated aquaculture is currently practiced in Canada and in China on pilot to commercial scales but it is not clear how this approach will develop with time. In the Mediterranean Sea, there are no commercial integrated aquaculture farms [21] and this is due to the fact that either the secondary crop is a low-value (not profitable) product or the secondary (extractive) crop is not able to grow in the oligotrophic conditions that characterize Mediterranean waters. The potential for integrated Mediterranean aquaculture exists, but it must be both ecologically and economically viable to work.

### Herbivorous Fish

One of the major challenges for both global and Mediterranean aquaculture is the limited supply of essential fish oil and fish meal [107]. The artificial diets of many farmed fish, including salmon, sea bream, and sea bass rely heavily on fish meal and fish oil, which places considerable pressure on wild fisheries (the source of fish meal and oil), severely jeapordizing the sustainability of the sector [108]. Several strategies have been proposed to address this problem, including the extraction of oils from fish-processing wastes [109, 110] and from fishery by-catch discards (the noncommercial fish

and animals that are caught by fishermen and subsequently thrown back to sea), and feeding fish with plant oils. There has been some success in the replacement of fish oils with plant oils [107], but many fish species have reduced survival and growth rates when reared without fish oils.

Another solution that has been proposed to address this problem is the rearing of herbivorous fish that do not require fish oils. Although these are generally not the highest value fish, they are nonetheless commercial species that are profitable to rear. The most common farmed herbivore in the Mediterranean is the diadromous gray mullet, *Mugil cephalus* (www.fao.org/fishery/culturedspecies/Mugil_cephalus/en). A lot of the pond rearing technology of this species was developed in Israel [111] and included polyculture. Egypt, the world leader in mullet production, has recently exceeded 1 million t/y. Although this fish is common in some of the southern Mediterranean countries, it does not have a large market in southern Europe and this is a challenge that needs to be overcome to promote herbivores as more sustainable species for aquaculture. Another problem that exists for *M. cephalus* is the absence of commercial hatcheries. Despite recent breakthroughs in spawning induction [112, 113], juvenile mullets are still collected from river mouths for aquaculture purposes thereby jeapordizing natural populations. These problems need to be addressed if this species is to be seriously considered a sustainable alternative to the common Mediterranean carnivores.

## Indicators for Sustainable Aquaculture

The Water Framework Directive establishes the Environmental Quality Standards for European waters, and all activities that may affect environmental quality, for example, aquaculture must comply with these standards. Aquaculture lease applications generally include Environmental Impact Assessments (EIA), which assess risks and predict the impacts of aquaculture. Monitoring is an approach to test if EIA predictions were correct, and to establish a feedback system to protect both the environment and the fish farmer. The Modeling-Ongrowing fish farms-Monitoring (MOM) system [114, 115] was developed for salmon farming in Scandinavia, and includes a feedback process of EIA – monitoring – farm adjustment. Although the MOM concept was developed for Scandinavian farms, this approach has been adopted by the operators of several farms in the Mediterranean Sea to monitor their performance and environmental status. Monitoring generally includes measurement of: (a) physical variables, such as hydrography, weather, water temperature, sediment type, etc.; (b) chemical variables, including dissolved oxygen, nutrients, suspended solids, dissolved and particulate organic matter, etc.; and (c) biological attributes, for example, algal pigments, biomass, productivity, macrofauna abundance, diversity, etc. Fernandes et al. [116] reviewed the science underlying aquaculture monitoring in Europe and found that it was generally motivated by research interests rather than by clear environmental objectives. Whereas comprehensive monitoring of marine environments improves the understanding of the functioning of these systems [117], and thus the ability to predict the response of these waters to anthropogenic perturbations, it is often not necessary to include many of the variables that are monitored [102].

The CONSENSUS project recently established a set of 18 indicators (www.euraquaculture.info/index.php?option%20=%20com_content&task%20=%20view&id%20=%20149&Itemid%20=%20118) to promote "European Best Aquaculture Practice." These indicators are currently being evaluated to examine their practicality and suitability for the sector. In a separate project entitled *ECASA* (www.ecasa.org.uk/), a set of indicators to assess aquaculture–environment interactions were evaluated in order to streamline the farm monitoring process. This was done for aquaculture in both northern European and several Mediterranean countries (e.g., [118]) yet despite the advances made in that project, there is still a need to further streamline the list of indicators. The main criteria that should be used as a guideline in the quest for optimal indicators have been described in UNESCO [119] and include: (a) relevance, (b) feasibility (amount of effort, expertise, and cost required to obtain the data), (c) sensitivity (to inform on how the environment is responding), and (d) clarity (how easy it is for stakeholders to understand). Although progress has been made toward developing the final list of such indicators for aquaculture, this work is only partially done and further work is needed to achieve this.

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# Marine Fisheries Enhancement, Coming of Age in the New Millennium

Kenneth M. Leber
Center for Fisheries Enhancement, Mote Marine Laboratory, Sarasota, FL, USA

## Article Outline



## Glossary

**Anadromous** Species that spawn in freshwater, then their offspring gradually make their way into estuaries or the sea, where they remain during much of the subadult and adult stages of the life cycle, before returning to rivers and streams to spawn.

**Catadromous** Species whose females release their eggs at sea, then the offspring move as larvae or early juveniles into estuaries, rivers, and streams where they spend the juvenile stage of the life cycle.

**Marine** Species that spawn in sea water, including those that spend most of their lives at sea and catadromous fishes, which spawn in seawater, then enter freshwater nursery habitats.

**Marine fisheries enhancement** Release of aquacultured marine organisms into seas and estuaries to increase or restore abundance and fishery yields in the wild.

**Outbreeding depression** Caused when offspring from crosses between individuals from different populations or subpopulations (stocks) have lower fitness than progeny from crosses between individuals from the same population/stock.

**Recruitment** The process of joining an existing population. Species *recruit* to the juvenile stages in nursery habitats; juveniles subsequently *recruit* to adult stages in adult habitats. Species *recruit* to a fishery when they reach the minimum size fished.

**Reintroduction** Temporary release of cultured organisms with the aim of reestablishing a locally extinct population.

**Restocking** Release of cultured juveniles into wild population(s) to restore severely depleted spawning biomass to a level where it can once again provide regular, substantial yields.

**Sea ranching** Release of cultured juveniles into unenclosed marine and estuarine environments for harvest at a larger size in "put, grow, and take" operations.

**Stock enhancement** The release of cultured juveniles into wild populations to augment the natural supply of juveniles and optimize harvests by overcoming limitations in juvenile recruitment.

**Supplementation** Moderate release of cultured fish into very small and declining populations, with the aim of reducing extinction risk and conserving genetic diversity. Supplementation serves primarily conservation aims and specifically addresses sustainability issues and genetic threats in small and declining populations.

## Definition of the Subject

Marine fisheries enhancement (aka "stock enhancement") is the use of hatchery-reared saltwater organisms to increase abundance and fishery yields in the wild. "Conservation hatcheries" also produce and stock depleted, threatened, or endangered organisms – to help preserve species in decline. The practice began in the latter part of the nineteenth century when fish hatcheries were first developed but understanding of the ecology and management of wild stocks into which the hatchery-reared organisms where released was very limited. Early stock enhancement thus has gone through a series of fits and starts and misfires. In the century after its birth, the technologies required for scientific inquiry of the effects and effectiveness of stocking hatchery-reared organisms were lacking. The science needed to guide reliable use of cultured

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aquatic organisms in conservation and resource management remained undeveloped. Then, at the close of the twentieth century, new mariculture, tagging, and genetic technologies surfaced and rapid advances were made in the science underpinning marine stock enhancement.

As growth in human population size approaches the carrying capacity of the planet in this century, and the world increasingly turns to the oceans to farm and harvest food [1], sustainable fishery yields and conservation of natural resources face unparalleled challenges. Over the past two decades, marine fisheries enhancement has been transformed from a tentative, poorly developed management tool to a maturing science. Some believe research funding for this field would be better spent on traditional fishery management. But today's seafood producers, fishery managers, and "...conservationists need all the tools that biology, ecology, diplomacy and politics can muster if endangered species are to survive beyond the next century," [2] and fisheries are to continue to support a viable seafood industry and sport pastime. This entry traces the emergence and progress of marine fisheries enhancement, and offers a prescription for future direction.

The term *stock enhancement* is originally derived from efforts to augment wild fish sub-populations, or "stocks," by releasing cultured fishes into aquatic environments. Stocking cultured organisms is one of the tools available for managing aquatic natural resources. It has been used with varying degrees of success to help increase abundance of habitat- or recruitment-limited stocks to help restore depleted populations, augment fisheries and help recover threatened or endangered species. There has been much debate over the effectiveness of stock enhancement as a fisheries management tool. However, most of the scientific evaluation of stocking is quite recent [3], as is a code of responsible practices that help guide effective application [4–6], and marine fisheries enhancement is finally poised for effective use.

In the USA, from the 1880s through the early 1950s, stocking hatchery-reared marine fishes was a principal approach used by the US Fish Commission (renamed Bureau of Fisheries in 1903, Bureau of Commercial fisheries in 1956, and later the National Marine Fisheries Service) for maintaining fishery stocks. But by the 1950s the practice of stocking marine fishes to manage US fisheries was curtailed for lack of evidence of its effectiveness in fisheries management [7]. Stocking was replaced by harvest management to control total catch and sustain fisheries. Stocking of freshwater habitats continued (particularly with salmonids into rivers), although the scientific basis for many of the management decisions needed for stocking salmonids was clearly lacking and did not begin to be addressed until the mid-1970s.

In the decade following 1975, scientists began to evaluate survival and fishery contributions of stocked salmon enabled by advances in fish tagging technology [8, 9]. Quantitative evaluation of marine fish stocking began in earnest in the 1980s and 1990s. The science underlying fisheries enhancement has since evolved to the point where, in some situations, stocking can be a useful fishery management tool to help restore depleted stocks and increase abundance in recruitment-limited fisheries [6]. Effective use of enhancement, though, requires full integration with harvest and habitat management, and a good understanding by stakeholders and resource managers of the opportunities where enhancement can be used successfully as well as its limitations [5, 6]. Principles for guiding the successful use of marine fisheries enhancement to help sustain aquatic resources are now being employed to design new enhancements and reform existing efforts. What follows is a brief overview of those principles and progress made in using hatchery-reared organisms to help sustain marine resources.

## Introduction

Marine fisheries enhancement is happening around the world and in some countries on a massive scale (e.g., China). However, in many countries the careful assessment of genetic and ecological risks is lagging behind implementation, putting wild stocks, the seafood supply, and sport fisheries at risk. The science of marine enhancement is still in its infancy compared to other fields of fisheries science, but now shows good potential to (1) increase fishery yield beyond that achievable by exploitation of the wild stock alone, (2) help restore depleted stocks, (3) provide protection for endangered species, and (4) provide critical information on the natural ecology, life history and environmental requirements of valuable marine species.

Stock enhancement has often been used as a generic term referring to all forms of hatchery-based fisheries enhancement. Bell et al. [3] and Lorenzen et al. [6] classified the intent of stocking cultured organisms in aquatic ecosystems into various basic objectives. Together, they considered five basic types, listed here from the most production-oriented to the most conservation-oriented:

1. Sea ranching – *recurring release of cultured juveniles into unenclosed marine and estuarine environments for harvest at a larger size in "put, grow, and take" operations*. The intent here is to maximize production for commercial or recreational fisheries. Note that the released animals are not expected to contribute to spawning biomass, although this can occur when harvest size exceeds size at first maturity or when not all the released animals are harvested.
2. Stock enhancement – *recurring release of cultured juveniles into wild population(s) to augment the natural supply of juveniles and optimize harvests by overcoming recruitment limitation in the face of intensive exploitation and/or habitat degradation*. Stock enhancements can increase abundance and fisheries yield, supporting greater total catch than could be sustained by the wild stock alone [10]. However, such increases may be offset, at least in part, by negative ecological, genetic, or harvesting impacts on the wild stock component. Stock enhancements tend to attract greater numbers of fishers, which can offset expected increase in each individual's catch-per-unit-effort (CPUE) [5, 11].
3. Restocking – *time-limited release of cultured juveniles into wild population(s) to restore severely depleted spawning biomass to a level where it can once again provide regular, substantial yields* [12]. Restocking requires release number to be substantial relative to the abundance of the remaining wild stock, and close ecological and genetic integration of wild and cultured stocks, combined with very restricted harvesting [6].
4. Supplementation – *moderate releases of cultured fish into very small and declining populations, with the aim of reducing extinction risk and conserving genetic diversity* [13, 14]. Supplementation serves primarily conservation aims and specifically addresses sustainability issues and genetic threats in small and declining populations [6].
5. Reintroduction – *involves temporary releases with the aim of reestablishing a locally extinct population* [15]. Continued releases should not occur, as they could interfere with natural selection in the newly established population. Fishing should also be restricted to allow the population to increase in abundance rapidly [6].

Scientific development of marine fisheries enhancement was lacking throughout most of the twentieth century. Although stocking cultured marine fishes began in the nineteenth century, the technology was limited to stocking only eggs and larvae. There were no published accounts of the fate of released fish until empirical studies of anadromous salmonids began to be published in the mid-1970s [16, 17], followed by the first studies (published in English) of stocked marine invertebrates in 1983 [18, 19] and marine fishes in 1989 [20].

During the past two decades, the field of marine fisheries enhancement has advanced considerably. Science in this field is rapidly growing, in part because of critical examination and debate about the efficacy of enhancement and the need for quantitative evaluation (e.g., [21, 22]), and in part because of advances made in aquaculture, genetics, tagging, and fishery modeling technologies, which have enabled quantitative studies and predictions of stocking effects. A clear process has emerged for developing, evaluating, and using enhancement [4–6]. Together, this process and the rapid growth of knowledge about enhancement effects should enable responsible and effective use of enhancement in marine fisheries management and ocean conservation.

## Scientific Development of Marine Fisheries Enhancement

### Scientific and Strategic Development

Since 1989, progress in marine fisheries enhancement has occurred at two levels – scientific advances and adoption of a careful and responsible approach to planning and organizing enhancement programs and manipulating abundance of marine species using aquacultured stocks. Much of the progress made in the 1990s was scientific and involved an expansion of field studies to evaluate survival of released fish

and improve the effectiveness of release strategies. The earliest studies on effectiveness of stocking *marine* fishes, published in English in the scientific literature, were in Japan [20, 23–26] and Norway [27–31], followed by studies in the USA [32–39], and Australia [40]. Progress made with invertebrates is well covered by Bell et al. [12].

Following the initial publications of scientific studies of marine fish enhancement, the number of peer-reviewed publications and symposia in this field began to escalate ([41–52], and see abstracts in [53]). It is now clear that stocking marine organisms can be an effective addition to fishery management strategies, but only when certain conditions are met. For stocking to be productive and economical, and help ensure sustainability of wild stocks, careful attention must be given to several key factors and stocking must be thoroughly integrated with fisheries management [6]. It is clear that stocking can be harmful to wild stocks if not used carefully and responsibly.

Aside from scientific gains in this field, the other level of progress made in the past two decades has been the evolution of a strategic "blueprint" for enhancements, such as the principles discussed in "a responsible approach to marine stock enhancement" [4, 6]. By the early 1990s, salmon enhancement in the US Pacific Northwest, which had been underway for a century, was beginning to incorporate reforms that were needed to improve efficiencies and protect wild stocks from genetic hazards that can lead to loss of genetic diversity and fitness. Concerns had been mounting over uncertainty about the actual effectiveness of salmon hatcheries and impacts on wild stocks. Concerns about wild stock impacts were twofold, including ecological effects of hatchery fish, such as competitive displacement, and genetic issues, such as translocation of salmon stocks, domestication and inbreeding in the hatchery and associated outbreeding depression, and loss of genetic diversity related to hatchery breeding practices (e.g., [54, 55]). Meanwhile, special sessions on marine stock enhancement began appearing at major fisheries and mariculture conferences in the early 1990s [41–44]. These sessions took a sharp turn from past approaches, where the principal focus in conference presentations about stock enhancement had been mainly on Mariculture research topics alone. The conveners of the special sessions on stock enhancement in the 1990s recruited presenters who worked on evaluating the effects and effectiveness of stocking hatchery organisms into the sea and interactions of hatchery and wild stocks. The special sessions focused on the "questions of the day" in marine enhancement and fostered debate in the marine enhancement research community about many of the reform issues being considered in salmon enhancement. The early 1990s was a period of rapid developments in enhancements, characterized by engagement of multiple scientific disciplines in a field that had previously been guided largely by a single discipline – aquaculture.

The salmon experience and reforms underway in salmon enhancement made it clear that a careful and multidisciplinary approach was needed in the development and use of marine enhancement. Many involved in developing new marine fisheries enhancement projects were paying close attention to the debate that had emerged over salmon hatcheries. Following the 1993 special session on "fisheries and aquaculture interactions" held at a mariculture conference in Torremolinos, Spain [44], several of the presenters (including scientists from Japan, Norway, the USA, and Italy [United Nations Food and Agriculture Organization, FAO]) met and formed an "International Working Group on Stock Enhancement," and affiliated the workgroup with the World Aquaculture Society. At that inaugural working group meeting, a decision was made to publish a platform paper to frame the question, "what is a responsible approach to marine stock enhancement?" This paper was presented at the 1994 American Fisheries Society symposium, "Uses and Effects of Cultured Fishes in Aquatic Ecosystems," and published in the 1995 peer-reviewed symposium proceedings [4]. The paper recommended ten principles for developing, evaluating, and managing marine stock enhancement programs. The Responsible Approach paper afforded a model for developing and managing new enhancement programs and refining existing ones. It has also helped frame research questions in the emerging science of marine fisheries enhancement.

The International Working Group on Stock Enhancement (IWGSE) was instrumental in advancing the science of marine fisheries enhancement in the 1990s. The working group focused primarily on

highlighting ongoing stock enhancement research around the world and fostering awareness of the Responsible Approach in their publications and presentations. International awareness and new research in the field was aided by the broad international makeup of the working group. Membership grew and soon included scientists from Australia, Canada, China, Denmark, Ecuador, Italy, Japan, Norway, Philippines, Solomon Islands, Spain, the UK, and the USA. Initially, the primary communication vehicle used by the working group was the special sessions on stock enhancement, which it planned and convened annually in various countries at the international conference of the World Aquaculture Society. The working group promoted a synergy among its members and the influence of the group expanded as members planned additional workshops and symposiums in their own countries and brought IWGSE scientists into the planning process.

The period 1990–1997 was a fertile time that gave birth to a rapid expansion of science in marine fisheries enhancement, which continues to this day, aided since 1997 in large part by the International Symposium on Stock Enhancement and Sea Ranching (ISSESR). The first ISSESR, held in 1997 in Bergen, Norway, was the brainchild of the Norwegian PUSH program (Program for Development and Encouragement of Sea Ranching) and the Norwegian Institute of Marine Research (IMR). In 1995, IMR scientists invited IWGSE scientists to become involved in the International Scientific Committee charged with planning the program for the first ISSESR. The first ISSESR, and the series of follow-up symposia that it launched (see www.SeaRanching.org), have encouraged and brought about fundamental advancements in the field of marine enhancement – by networking the scientists working in this specialized field, highlighting their work at the ISSESR, and publishing their peer-reviewed articles in the symposium proceedings. The 3–5 day ISSESR has now become a regular scientific symposium event, hosted by a different country every 4–5 years. Following the first ISSESR in Bergen [47], subsequent symposiums in the series were held in Kobe, Japan in 2002 [49], in Seattle, USA in 2006 [52], and in Shanghai, China in 2011 [53]. The fifth ISSESR will be held in Sydney, Australia in 2015 or 2016. Inquiries from scientists in different countries interested in hosting the sixth one are already being received by the organizing group. Following the first ISSESR, the IWGSE scientists continued the efforts they started in the working group through their involvement in the International Scientific Committees for the ISSESR and steering committees for other stock enhancement symposia (e.g., [46, 48, 51]). In 2010, a refined and updated version of the Responsible Approach was published [6] and presented at the fourth ISSESR.

As in any new science, lack of a paradigm and consensus on the key issues retard progress. The ISSESR and other marine enhancement symposia and working groups have helped to place scientific focus on critical uncertainties and communicate results of new science in this field at symposiums and in the scientific literature. They have also provided a forum for debate on the issues, and increased networking of scientists, resource managers, students, and educators working in this field worldwide. The focus on key issues is nurturing this new field of science.

## Technological and Tactical Constraints

Although marine enhancements do show promise as an important tool in fisheries management, why has this field taken so long to develop and why have marine enhancement programs often failed to achieve their objectives? The scientific development of marine fisheries enhancement has long been impeded by lack of the technologies needed to evaluate effects of stocking cultured fish. Although marine enhancements began in the 1880s, until the advent of the coded-wire tag in the mid-1960s [8], there was no way to identify treatment groups and replicates in experimental releases of juvenile cultured fish [56]; and quantitative marking methods for multiple experimental groups of postlarvae and very small juveniles (<50 mm in length) came much later (e.g., [57]). To make matters worse, scientific development of marine enhancement was also stymied by lack of adequate technology for culturing marine fishes. Rearing methods for larval and juvenile marine fishes, many of which require live feeds during the larval stage, remained undeveloped until the mid- to late 1970s, when breakthroughs finally began to be achieved in rearing a few marine species past metamorphosis [58]. By the mid-1980s mass production of juveniles had been achieved for several

species of marine fishes. Even today, though, many marine fishes cannot yet be cultivated to the juvenile stage in the quantities needed for stocking. Without the availability of juveniles grown to a wide range of sizes, fundamental questions about density dependence, hatchery-wild fish interactions and cost-yield efficiency of size-at-release and other release variables cannot be addressed in field experiments. Thus, even the basic technologies needed to develop and understand the potential of marine enhancement have been unavailable until relatively recent times for some fishes and have yet to be developed for others.

Technology has not been the only constraint to successful development of marine fisheries enhancement. The effective use of stocking cultured marine organisms in fisheries management has been hindered by lack of understanding of the effect of releases on fish population dynamics and a lack of related, quantitative assessment tools [10]. Moreover, there has been a lack of essential governance and fisheries management considerations in planning, designing, implementing, and evaluating enhancement programs [6, 59]. A symptom of this is the relentless concern among stakeholders and hatchery managers alike about the numerical magnitude of fish released, rather than on the effective contribution of the hatchery program to fisheries management goals. Certainly, a hatchery needs to meet some release quotas, but the numbers of fish released is a misleading statistic for gauging success or comparing effectiveness among enhancement programs. Yet, from the very beginning, progress has been judged by the number of eggs, yolk-sac larvae or juveniles stocked, rather than by the number of fish added to the catch or to spawning stock biomass. The thinking behind this approach apparently is "grow and release lots of hatchery fish and of course they'll survive and add to the catch," without realizing the need to optimize release strategies (e.g., [39, 60, 61]) (e.g., to know what size-at-release, release habitat and release magnitude combination has the greatest impact on population size, fishery yields, and economics), or that the impact from stocking could in fact be a negative one on wild stocks (such as replacement of wild fish by hatchery fish) if certain precautions are not taken. This attitude has been pervasive and exists even today among many stakeholders and enhancement administrators. In fact, research now shows that survival and recruitment to the fishery following hatchery releases is a complex issue that requires much greater understanding about the fishery, hatchery fish performance, and biological and ecological factors in the wild than simply "the catch is down, thus releasing large numbers of fish will bring it back up." And quite often large release magnitudes are achieved by releasing millions of postlarvae, rather than fewer but larger juveniles. But releases of postlarvae alone may be effective, yet can also be totally ineffective, depending on conditions at the release site [62].

The key to successful use of stocking is to plan enhancement programs from a fisheries/resource management perspective, using a broad framework and scientific approach [6, 59]. The probability of achieving effective results is greatly increased when stakeholders are engaged from the outset in planning *new* programs, using a framework that is structured, multilayered, participatory, and makes good use of science, to design, implement, and analyze enhancement fisheries systems [6]. Incorporating the key principles in the Responsible Approach into the frameworks of *existing* programs as well is likely to improve performance.

## Responsible Approach to Marine Fishery Enhancement

In retrospect, the slow development of marine fish culture (a century behind salmonid aquaculture) has helped marine stock enhancement programs avoid some of the mistakes of the past made with salmon stock enhancement, where lack of understanding of genetic issues during most of the twentieth century led to inadvertent domestication and inbreeding in salmon hatchery populations, leading to reduced fitness in wild stocks. Marine finfish juvenile production technology lagged behind freshwater and anadromous fish culture by a century. Thus, mass release into the sea of juvenile marine fishes large enough to survive and enter the breeding population did not begin until the 1980s. The relatively recent capabilities to conduct marine fisheries enhancement emerged at about the same time that geneticists realized that hatchery practices with salmonids (1) could reduce genetic diversity in the hatchery and ultimately, enhanced wild stocks, owing to inadequate broodstock management, (2) have caused translocations of salmon genes into

environments where they are less fit, and (3) have contributed to loss of local adaptations in the wild population. Today, population genetics is much better understood and broodstock genetics and hatchery practices can be better managed to address these concerns (e.g., [63–65]). Thus, marine enhancement programs need careful guidance from qualified geneticists. The Puget Sound and Coastal Washington Hatchery Reform Project in the USA has been instrumental in reforming salmon enhancements [66]. This group affords a model for managing enhancement hatcheries in the twenty-first century.

As progress was being made in the early 1990s to better understand the genetic structure of stocks and how to manage genetics in hatcheries, realizing the need for reform in approaches to enhancing non-salmonids was just beginning. In the mid-1990s, Cowx [67], for enhancements in freshwater systems, and Blankenship and Leber [4], for enhancements in marine and estuarine systems, published papers calling for a broader, more systematic, reliable, and accountable approach to planning stock enhancement programs. Prompted both by the salmonid hatchery reform movement and by the WAS IWGSE, the ten principles presented in Blankenship and Leber ([4] Table 1) gained widespread acceptance as the "Responsible Approach" to stocking marine organisms and provided a platform for subsequent discussions on planning, conducting, and evaluating marine enhancements (e.g., [6, 12, 22, 51, 52, 68–70]). Since 1995, the awareness of the Responsible Approach has steadily increased and has helped guide hatchery and reform processes for marine enhancements worldwide [11, 36, 37, 39, 60, 62, 69–90].

The Responsible Approach provides a conceptual framework and logical strategy for using aquaculture technology to help conserve and increase natural resources. The approach prescribes several key components as integral parts of developing, evaluating and managing marine fisheries enhancement programs. Each principle is considered essential to manage enhancements in a sustainable fashion and optimize the results obtained [4, 6].

A major development since the publication of the original "Responsible Approach" has been increasing interest from fisheries ecologists in understanding and quantifying the effects of hatchery releases from a fisheries management perspective. This has led to the development of fisheries assessment models that can be used to evaluate stocking as a management option alongside fishing regulations [5, 10]. At the same time, approaches to fisheries governance underwent major changes that allow enhancements to become more integrated into the management framework and in some cases, were driven by interest in enhancement approaches [59].

Walters and Martell [5] discuss four main ways that a marine enhancement program can end up causing more harm than good: (1) the replacement of wild with hatchery recruits, with no net increase in the total stock available for harvest (competition/predation effects); (2) unregulated fishing-effort responses to the

**Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 1** The ten principles of a responsible approach to marine stock enhancement [4]

| | |
|---|---|
| 1 | Prioritize and select target species for enhancement by ranking and applying criteria for species selection |
| 2 | Develop a management plan that identifies how stock enhancement fits with the regional plan for managing stocks |
| 3 | Define quantitative measures of success to track progress over time |
| 4 | Use genetic resource management to avoid deleterious genetic effects on wild stocks |
| 5 | Implement a disease and health management plan |
| 6 | Consider ecological, biological, and life history patterns in forming enhancement objectives and tactics; seek to understand behavioral, biological, and ecological requirements of released and wild fish |
| 7 | Identify released hatchery fish and assess stocking effects on the fishery and on wild stock abundance |
| 8 | Use an empirical process for defining optimal release strategies |
| 9 | Identify economic objectives and policy guidelines, and educate stakeholders about the need for a responsible approach and the time frame required to develop a successful enhancement program |
| 10 | Use adaptive management to refine production and stocking plans and to control the effectiveness of stocking |

**Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 2** Code of responsible conduct for marine stock enhancement [5]

| |
|---|
| • Make certain that management priorities and acceptable trade-offs are absolutely clear |
| • Do careful stock assessments to show that the target stock is recruitment overfished or can no longer rear successfully in the wild |
| • Show that enhanced fish can recruit successfully in the wild |
| • Show that total abundance is at least initially increased by the hatchery fish contribution |
| • Show that fishery regulations are adequate to prevent continued overfishing of the wild population, unless there has been an explicit decision to "write off" the wild population |
| • Show that the hatchery production system is actually sustainable over the long run, when it is to be a permanent component of the production system |

presence of hatchery fish that cause overfishing of the wild stock; (3) "overexploitation" of the forage resource base for the stocked species, with attendant ecosystem-scale impacts; and (4) genetic impacts on the long-term viability of the wild stock. They stress that it is critical to monitor the impacts of enhancement as the program develops to have evidence in hand if debate about the efficacy of the program does surface. To help guide developing programs, they provide and discuss a "Code of Responsible Conduct" as critical steps in marine fisheries enhancement program design (Table 2).

In 2010, Lorenzen, Leber, and Blankenship [6] published an updated version of the Responsible Approach to refine the original key principles and include five additional ones (Table 3). The key principles added in the updated version bring stakeholders more firmly into the planning process; place much stronger emphasis on a-priori evaluation of the potential impact of enhancements using quantitative models; place marine fishery enhancements more firmly within the context of fishery management systems; emphasize design of appropriate aquaculture rearing systems and practices; and incorporate institutional arrangements for managing enhancements. Lorenzen et al. [6] provide comprehensive discussions for each of the 15 key

**Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 3** The updated responsible approach (From [6])

| | |
|---|---|
| Stage I: Initial appraisal and goal setting | |
| 1 | Understand the role of enhancement within the fishery system [*new*] |
| 2 | Engage stakeholders and develop a rigorous and accountable decision making process [*new*] |
| 3 | Quantitatively assess contributions of enhancement to fisheries management goals |
| 4 | Prioritize and select target species and stocks for enhancement |
| 5 | Assess economic and social benefits and costs of enhancement |
| Stage II: Research and technology development including pilot studies | |
| 6 | Define enhancement system designs suitable for the fishery and management objectives [*new*] |
| 7 | Design appropriate aquaculture systems and rearing practices [*new*] |
| 8 | Use genetic resource management to maximize effectiveness of enhancement and avoid deleterious effects on wild populations. |
| 9 | Use disease and health management |
| 10 | Ensure that released hatchery fish can be identified |
| 11 | Use an empirical process for defining optimal release strategies |
| Stage III: Operational implementation and adaptive management | |
| 12 | Devise effective governance arrangements [*new*] |
| 13 | Define a management plan with clear goals, measures of success, and decision rules |
| 14 | Assess and manage ecological impacts |
| 15 | Use adaptive management |

principles listed in Table 3. Readers are urged to consult Lorenzen et al. [6] for additional detail, as it is beyond the scope, here, to repeat their discussions of each principle.

The 15 principles in the updated Responsible Approach include the broad range of issues that need to be addressed if enhancements are to be developed or reformed responsibly [6]. Clearly, marine

**Marine Fisheries Enhancement, Coming of Age in the New Millennium. Table 4** Key areas of expertise needed in marine fisheries enhancement

| |
|---|
| • Fisheries science |
| • Fisheries management |
| • Adaptive management |
| • Marine aquaculture |
| • Population genetics |
| • Aquatic animal health |
| • Population ecology |
| • Behavioral ecology |
| • Community ecology |
| • Resource economics |
| • Social science and institutional analysis and design |
| • Statistics and experimental design |
| • Tagging technology |
| • Communications and outreach |

enhancement programs are multidisciplinary and their effective use requires specialist knowledge and skills from diverse fields (Table 4). Forming interdisciplinary teams of the various specialists required is an important factor in employing the Responsible Approach in developing, reforming, and executing marine enhancements. For effective design of enhancement programs, specialists in each area of expertise listed in Table 4 should be included in the planning teams.

It should be clear that without a careful monitoring system in place, marine enhancements simply cannot be managed. Monitoring is essential to understand the impacts of enhancement, to manage release strategies so that they are efficient and designed well enough to achieve the goals of the program, to protect against misuse of stocking (as discussed in 5 and 6), resulting in harm to wild stocks, and to document success or failure in meeting enhancement program objectives. Walters and Martel [5] list several key monitoring requirements for managing fishery enhancements well: (1) mark all (or at least a high and known proportion of) fish released from hatcheries; (2) mark as many wild juveniles as possible at the same sizes/locations as hatchery fish are being released; (3) experimentally vary hatchery releases over a wide range from year to year and from area to area, probably in on/off alternation (temporal blocking) so as to break up the confounding of competition/predation effects with shared environmental effects; (4) monitor changes in total recruitment to, production of, and fishing effort in impacted fisheries, not just the percentage contribution of hatchery fish to production; (5) monitor changes in the fishing mortality rates of both wild and hatchery fish directly, through carefully conducted tagging programs that measure short-term probabilities of capture; and (6) monitor reproductive performance of hatchery-origin fish and hatchery-wild hybrid crosses in the wild. Sound management-action design and monitoring is the essence of adaptive management [91] and adaptive management enables refinements, progress, and success in marine enhancement programs [4, 6, 11, 92].

Marine fisheries enhancement is a powerful tool that requires careful and interdisciplinary planning to control its effects. The process of transforming marine enhancement from an idea before its time into an effective resource management and sea ranching tool involves adopting a clear prescription for responsible use. As marine enhancement comes of age in this new millennium, agencies and stakeholders have a growing library of protocols for enhancement at their disposal and the responsibility to use them. The Responsible Approach and Code of Responsible Conduct provide healthy prescriptions for controlling the outcome of enhancements. These principles need to be adopted and used well, in order to increase and ensure the readiness of this tool to aid in conservation and to increase fishery yields when it is needed. Growth in human population size is fast approaching a critical level, and much greater attention will be placed in this century on obtaining food from the sea [1]. It is unwise to not be ready with marine enhancement to help sustain depleted, threatened, and endangered species, help maintain wild stocks in the face of increasing fishing pressure, help sustain sports fisheries, and help increase fishery yields.

## Legacy from the Past

### Allure of a Quick Fix

Marine enhancement programs are often seen as a "quick fix" for a wide variety of problems in marine

resource management. At best, they may be an important new component of marine ecosystem management; if not implemented responsibly, though, they may lull fishery managers into false confidence and thus lead to inaction and delay in the development of other fisheries management and restoration programs [5, 6].

Although marine fisheries enhancement is certainly not a quick fix, it can be a powerful tool for resource management when conditions warrant the use of this tool and if the time and care needed are taken to develop enhancement programs well. Unfortunately, the allure of a quick fix has often prompted stakeholders and managers to skip or ignore several elements needed to allow those programs to succeed, leading to wholesale failure of such efforts. The field of marine fisheries enhancement is littered with examples of enhancement projects that failed to achieve their potential for lack of a careful enough or quantitative approach (e.g., see accounts discussed in [7, 21, 62, 72, 93–95]). Most of the failures can be traced back to attempts to use enhancements when they were not warranted or failure to consider several, if not most, of the principles now incorporated in the "Responsible Approach" and "Code of Responsible Conduct" for marine fisheries enhancement.

### Isolation from the Fisheries Science Community

Historically, marine fisheries enhancements have been conducted more or less isolated from other forms of fisheries management. Enhancement hatcheries have often been promoted by stakeholders and government mandates without the necessary funding or authorization behind them to do much more than produce and release fish without funds for monitoring impacts and adaptive management needed to increase the effectiveness of enhancements. Such programs are often built and implemented from a vantage point within resource management agencies that has little or no connectivity with the existing fishery management process. This has stymied development of this field in two ways – first, by compelling hatcheries to operate within resource management agencies largely independent from stock assessment and fisheries monitoring programs, or even worse, within different agencies altogether. Second, such isolation has fostered development of a production-oriented operational mode, and thwarted development of an enhancement-oriented mode [92].

Part of this isolation from fishery management also stems from the poor track record of the early marine hatcheries as an effective way to recover depleted fish stocks, coupled with the lack of scientific development of marine fisheries enhancement for so long into the twentieth century. This has understandably led to bias against fishery enhancements. Many of today's fishery scientists have been schooled to understand that stock enhancement has not worked, based in part on the lingering legacy from past failures and in part on lack of awareness of new marine fisheries enhancement science, as few citations have yet appeared in fisheries science textbooks. With many of the scientific achievements in fisheries enhancement having occurred only over the past decade or so, this is understandable. But in light of the need to couple fisheries enhancement with fisheries management systems, lack of awareness of progress in this field is an obstacle that may be resolved only by compilation of more and more success stories over time. Thus, it is imperative that existing and developing enhancement programs alike incorporate modern concepts about how to plan and conduct enhancements so they are enabled for success.

## Progress in Marine Fisheries Enhancement

### Lessons Learned from Marine Enhancement Programs

Much progress has now been made in understanding how to manage enhancement more effectively. Bartley and Bell [96] considered progress made from three decades of stocking initiatives and summarized and discussed lessons learned. These are listed here, below [96], with a brief clarification or caveat on each.

#### Deciding When and How to Apply the Release of Cultured Juveniles

1. Objective assessment of the need for releases is crucial – and requires an evaluation of the status of the fishery, modeling of stocking impact to determine if stocking can help achieve the goals, coupled with consideration of whether there are recruitment limitations and adequate habitat available for stocking.

2. Releases of cultured juveniles for restocking and stock enhancement need to be made at the scale of self-replenishing populations – releases will not be effective unless the spatial extent of target populations has been identified; thus prior to conducting releases of hatchery organisms, clear identification of genetically discrete stocks should be determined.
3. There are no generic methods for restocking and stock enhancement – largely because of wide variation in life history among different species and variation in ecological conditions among release sites.
4. Very large numbers of juveniles are often needed for effective stock enhancement – this is particularly so for offshore stocks, which can be comprised of a huge number of individuals; more modest releases may suffice for localized enhancement of inshore stocks or those comprised of multiple stocks that occur on relatively small scales.
5. Large areas are needed for stock enhancement of some species – and this can result in user conflict, particularly for sea ranching, where large areas are leased and protected by the enhancement program (e.g., [97]); in other cases, limited dispersal of adults and larvae indicates stocking in smaller areas can be effective, for example, common snook along Florida's Gulf Coast [98].
6. Invertebrates offer good opportunities for restocking and stock enhancement – because invertebrates are often comprised of self-recruiting populations that occur at small scales.

### Integrating Interventions with Other Management Measures

7. Problems that caused lower production must be addressed before release of juveniles – particularly in the case of degraded, lost, or insufficient habitat. With better management of the wild resources, the scope for augmentation of total production declines; enhancement becomes a very site specific tool when habitat has been lost, or something needs rebuilding, or there are species of particularly high value [94].
8. Biotechnical research must be integrated with institutional and socio-economic issues – ownership rights and control and use of enhanced stocks need to be well understood by the greater institutional, social, economic, and political environment [99].
9. Successful stock enhancement programs are often run by cooperatives and the private sector – where there is increased incentive in sharing the costs of fisheries enhancement.
10. The costs and time frames involved in restocking programs can be prohibitive – hatchery costs, which can be considerable, are particularly difficult to bear in smaller countries and developing countries.

### Monitoring and Evaluation

11. Development of cost-effective tagging methods is critical to efficient evaluation of stock enhancement – refining and monitoring the effects and effectiveness of marine enhancements cannot be done without a way to distinguish hatchery from wild stocks and distinct release groups.
12. Large-scale releases of hatchery-reared juveniles can affect genetic [fitness] of wild populations – genetic hazards can be caused by hatchery-wild fish interactions and these need to be minimized.

### Reducing the Cost of Juveniles

13. Costs of stocking programs can be reduced by "piggybacking" production of juveniles for release on existing aquaculture – this could reduce or eliminate the need for expensive new hatchery construction for enhancement programs, as long as appropriate broodstock management protocols are in place for conserving wild-stock genetics.
14. Wild [postlarvae] can provide an abundant, low-cost source of juveniles for stock enhancement programs – this can sometimes be an effective way to reduce costs and eliminate genetic issues; successful scallop enhancement in Japan is based on collection of wild seed stock.
15. The costs of restocking can be reduced greatly for some species by relocating adults to form a viable spawning biomass – rebuilding spawning aggregations by concentrating broodstock can be effective for depleted stocks with limited larval dispersal, but care must be taken to avoid comingling different stocks (i.e., avoid translocation of exogenous genes).

#### Improving Survival in the Wild

16. Predation is the greatest hurdle to survival of released juveniles – care must be taken to understand ecology of the species and ecosystem at the release site and pilot experiments are needed to develop optimal release strategies to maximize survival.
17. Excessive releases of juveniles cause density-dependent mortality – density has a strong effect on growth and survival in the wild; planning release magnitude must take into account the carrying capacity at release locations. This requires adaptive management and an experimental framework for releases.
18. Small-scale experiments to test methods for releasing juveniles can give misleading results – "commercial scale" releases are needed to test assumptions made from small-scale release experiments.
19. Good survival of released juveniles at one site is no guarantee that the methods can be transferred to other sites – stocking effectiveness will vary with release location and what works at one site may not be effective at another.

#### Other Manipulations to Increase Abundances

20. Artificial habitats can be used to increase the carrying capacity for target species – and may enable increased production at release sites where there are resource (food, refuge, space) limitations.
21. Yields of some species can be increased by providing suitable settlement habitat and redistributing juveniles from areas of heavy settlement – for example, redistribution can be used to reduce density effects and increase probability of successful recruitment when moved to a location with greater availability of food, refuge, or settlement habitats. But care must be taken to avoid genetic hazards associated with comingling stocks.

## Examples of Progress Made in Marine Enhancement

As science and constructive debate have advanced in this field, there are many signs of progress. Some explicit examples of progress made in marine enhancement over the past couple of decades are presented below, ranging in scale from local experimental investigations of release strategies and density-dependent effects on hatchery and wild stocks (e.g., [100]) to documented replenishment impact in large-scale enhancement efforts (e.g., [101, 102]). This is but a sample of examples and is by no means a comprehensive list. There are many more examples in the peer-reviewed proceedings from the ISSESR and other stock enhancement conferences [41–53] and other journal articles.

1. Adoption of a science-based responsible approach to marine stock enhancement has now become widespread, resulting in a much more assessment-driven and precautionary approach than ever before (a few examples include Refs. [4, 6, 10, 12, 20, 22, 27–29, 33, 37–39, 59–61, 68, 69, 72, 75, 84, 86, 87, 89, 96, 103–106]). This has been enabled, in part, by advances in tagging technology (e.g., [8] and see examples in [9, 56]) and in development of new marine aquaculture technologies that can now provide juvenile fishes for marine enhancement research.
2. Networking of Scientists involved in this rapidly advancing field has been fostered by various symposia and working groups, for example, the World Aquaculture Society Working Group on Stock Enhancement and the scientific committees for the International Symposium on Stock Enhancement and Sea Ranching (www.SeaRanching.org).
3. There is a much better appreciation of the importance of managing marine fishery enhancements from a fisheries management perspective (e.g., [6, 59, 107]).
4. New tools are available for modeling stock enhancement effects and effectiveness [10, 82, 108–110].
5. At least two experimental field studies have now been conducted to evaluate density-dependent interactions of stocked hatchery and wild fish; these provide evidence that increased production can be achieved in juvenile nursery habitats without displacing wild fish, but not necessarily without displacing some of the hatchery fish [33, 100].
6. There is now clear evidence and a prescription of techniques for improving post-release survival (often with a doubling effect or more) of stocked marine fishes, and optimizing release strategies to maximize stocking efficiency and control impacts

(e.g., [26, 36, 37, 39, 60–62, 70, 72, 100–115]). There is also ample evidence that in habitats with limited carrying capacity or intense predation, regardless of release strategy used, little can be done to improve survival of hatchery fish and stocking simply cannot increase production [106, 116, 117].

7. It is now fairly clear that marine enhancements may be cost effective only if (a) the supply of recruits is generally limiting, (b) there is adequate habitat to support an increased supply of juveniles, (c) cultured juveniles represent a large portion of recruitment, (d) fishing is regulated appropriately, and (e) other management measures (catch regulations and habitat restoration) are insufficient to restore catch rates [96].
8. Stock enhancement of some species of marine finfish has been successful at the scale of large bays, for example, Hirame flounder and red sea bream in Japan [72, 106] when there is sufficient carrying capacity at release sites. Carrying capacity varies considerably among release sites, and thus must be evaluated and taken into account using monitoring and adaptive management for each release site.
9. Scallop sea ranching has been a large success in Japan, New Zealand, and China, where property rights and large ocean leases have created strong incentives for careful management by fishermen and owners of the sea ranching operations [72, 101, 102, 118]. For example, near Dalian, China, Zhangzidao Fishery Group leases 2,000 $km^2$ of ocean-bottom-to-ocean-surface for sea ranching. In 2010, Zhangzidao harvested an average of 150 t/day of ocean scallops from their sea ranching operations (over 50,000 t/year) (Wang Qing-yin, personal communication 2011).
10. Property rights have also provided incentives for bivalve culture in the State of Washington, USA, where clam sea ranching operations have remained economically and environmentally sustainable for over three decades [119].
11. Pilot experiments with black bream in an Australian estuary have documented quite good survival and recruitment to the fishery. The latest phase of this project reveals strong rationale for long-term monitoring of enhancement impact [87, 120].
12. Restocking success with red drum in a South Carolina estuary [77, 121]. Pilot experiments revealed surplus productive capacity in the Ashley River in South Carolina, where fishery landings of red drum were doubled over a few years.
13. Pilot experiments to evaluate blue crab enhancement potential in Maryland and Virginia led to improvements in traditional fishery management, with information learned through stocking research [70, 114]. Pilot experiments can be used to provide critical information on the natural ecology, life history, and environmental requirements of valuable marine species [122].
14. Perhaps the largest scale enhancement success for fishes is Japanese chum salmon restocking – a special tool for a circumstance in which the habitat had almost totally been lost [94].

## Future Directions

Over the past two decades, there has been a rapid expansion of knowledge about marine fisheries enhancement systems and the effects and effectiveness of stocking a wide variety of marine organisms for sea ranching, stock enhancement and restocking. Many gaps in knowledge have now been filled. Well thought out approaches now provide a roadmap for effective use of enhancements. When models show potential for stocking, efforts to deploy marine enhancements can be successful if the principles in the roadmap are carefully employed. The basic reason that marine enhancement programs do not have more of a track record of success stories yet is that implementing them well is a complex endeavor that demands attention to multiple factors spanning many disciplines. Rarely have these been pulled together in an enhancement program. The Hatchery Reform Project in the Pacific Northwest USA, which includes an independent scientific review panel ("Hatchery Scientific Review Group") is a good example [123]. Because of their efforts, salmonid hatchery reforms now underway are bringing many of the principles of the Responsible Approach into play. The Norwegian PUSH program is another good example. In that case, information gained from quantitative assessments of enhancement showed that stocking would not be an economical way to enhance cod in Norway, thus saving years of wasteful

spending that could have occurred there, had monitoring and adaptive management not been a central part of the enhancement system.

Successful examples of fisheries enhancement are truly group efforts, involving stakeholders, agency officials, and individuals with expertise in the principal sub-disciplines needed. Suffice to say that at this point in time few, if any, marine fisheries enhancement programs have enlisted all of the key elements of the Responsible Approach and Code of Responsible Conduct. But these principles are now well described and laid out in a systematic manner. It is reasonable to expect that if the Responsible Approach is used as the blueprint for planning and executing enhancements, and if the initial appraisal and goal setting stage indicates moving ahead, then there is ample opportunity for success in applying marine fisheries enhancements, as long as dedicated attention is focused on applying each of the key elements.

So how will marine enhancement advance to the next level – emergence of a rapidly growing body of success stories in restocking, stock enhancement, and sea ranching? Listed below are a few factors that are now needed to transition this field to the next level, where marine enhancements are well integrated into resource management systems and used wisely and appropriately.

## Enabling Factors for Increasing Successful Marine Enhancements

1. *Greater awareness is needed among all stakeholders of the issues, pitfalls, progress, and opportunities in this field.* The concepts underlying effective enhancements need to be translated into lay language and used to inform stakeholders. This will help all stakeholders recognize the various issues and parameters needed for effective enhancements. Pivotal among stakeholders are public officials who fund enhancement programs, as they need to understand what it takes to develop an effective program or reform existing ones. New enhancement programs that may not be funded well enough to implement all of the key principles in the Responsible Approach would do well to use the results of Stage 1 in Table 3 to document the potential for success, but not proceed beyond Stage 1 until adequate funding is available.
2. *Use of Adaptive management is one of the most important principles for guiding successful enhancement programs.* Active adaptive management [91] is critical for gauging the effectiveness of, improving, and managing fisheries systems in the face of uncertainty. However, it is often dismissed by enhancement programs or given low priority for lack of funding or when enhancement is viewed as a quick fix. But, this important principle is used to optimize release strategies, to identify and deal with ecological or genetic impacts on wild stocks, to refine the enhancement process and identify the results of improvements, to evaluate and improve progress towards goals and objectives, and to monitor and improve economic impact. Active adaptive-management is an essential component of managing enhancement programs; it empowers management teams to understand and control the impacts of enhancements well. Without it, enhancement programs at best rely on hope to achieve their potential (but cannot) and at worst are doomed to failure. Australia is employing active adaptive management principles early in the development stage as part of ongoing work to evaluate enhancement potential for a wide range of species [124].
3. *Adapt the Responsible Approach to local circumstances.* The Responsible Approach is purposely vague on how to implement it. This is partly because not all elements are needed under all situations, but most will be. Fitting the process to particular circumstances is in itself a key part of implementing the Responsible Approach by engaging the various stakeholders in planning [6]. As progress continues in this field, additional principles will emerge that need to be included, for example, to account for needs of regional fishery management plans in response to climate change.
4. *Seek assistance from established workers in the field.* For new and developing enhancement programs, or existing ones seeking to design and implement reforms, there is a broad and expanding network of workers in this field who could be queried for advice on various enhancement issues. The ISSESR website is a good source for identifying individuals with specific kinds of expertise, by perusing presentation abstracts or locating published proceedings from past ISSESR conferences [125]. If researchers

or workers in the field are contacted, but do not have time to provide advice, they usually will help identify others who can.

This entry may help expand awareness among fishery stakeholders, other natural-resource stakeholders, scientists, and fishery managers alike about the pitfalls, challenges, and progress made in using marine hatchery releases as one of the tools in resource management and seafood production. Readers are referred to the articles and symposium proceedings cited herein to gain a better understanding of the issues, lessons learned, and progress.

The debate focused on enhancement is a healthy one, for it is fostering steady improvements and reforms in existing programs, and careful planning and design in new ones. With each advance made, the potential seen by our forefathers to use hatcheries as a tool for recovering depleted stocks, increasing abundance in recruitment-limited stocks, and producing seafood by sea ranching is coming closer to fruition. One of the greatest lessons learned from the past is that the emphasis on expanding hatchery fish production for marine enhancement should not be allowed to take the focus off of the objective – increasing yields in fisheries and recovering stocks in restoration programs. Clearly, marine fisheries enhancement is a strong tool to add to the fishery management toolbox. But only careful analysis of conditions of the wild stock and the fishery will guide when and where it is appropriate to use enhancements in addition to other management options, and when to stop. As Albert Einstein once said, "a perfection of means, and confusion of aims, seems to be our main problem." With the focus shifted to outcomes in marine enhancement programs, the appropriate means should fall into place, aided by healthy debate and prescriptions for a responsible approach to marine fisheries enhancement.

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# Marker-Assisted Breeding in Crops

Roberto Tuberosa
Department of Agroenvironmental Science and Technology, University of Bologna, Bologna, Italy

## Article Outline



## Glossary

**Backcross** Procedure used by plant breeders to introgress an allele at a locus of interest (e.g., disease resistance) from a donor parent to a recurrent parent, usually a successful cultivar. The recurrent parent is crossed several times to the original cross and selection is performed at each cycle to recover the plants with the desired allele and the largest portion of the genome of the recurrent parent.

**Candidate gene** A coding sequence that is supposed to be causally related to the trait under selection. The candidate-gene approach is best applied with simple biochemical traits when a clear cause-effect relationship can be established between the gene function and the target trait.

**Epistasis** The interaction between two or more genes to control a single phenotype. Interaction between two or more loci that control the same trait. The presence of epistatic loci makes it more difficult to predict the phenotypic value of progeny derived either from crosses or from selfing.

**Forward genetics** Approaches to dissect the genetic makeup of traits starting from the observation of the phenotype. QTL mapping and positional cloning are examples of forward genetics to investigate quantitative traits.

**Haplotype** Chromosome fragment of varying length carrying a common set of marker alleles in close linkage at adjacent loci. When using haplotypes in association mapping studies, the information of several linked bi-allelic markers is combined as a single, multi-locus informative marker.

**Heritability** The portion (from 0% to 100%) of phenotypic variability that is genetically determined. The additive portion (i.e., not due to dominance) of variability is inherited from one generation to the next and is the main determinant of the gain from selection. Heritability is specific to a particular population in a particular environment.

**Introgression library lines (ILLs)** A collection of lines (ca. 80–100) obtained by subsequent backcrosses of a recurrent parent (usually an elite cultivar) with a donor parent, usually a line highly diversified from the recurrent parent for one or more traits. Each ILL carries a fragment (from ca. 20 to 40 cM) of the donor genome different from that carried by the other lines. Collectively, the fragments of all ILLs cover the entire genome with partial overlap. ILLs are ideal for the fine mapping and cloning of major loci and to investigate epistatic interactions.

**Linkage disequilibrium (LD)** The level of nonrandom assortment of alleles at different loci. The level of LD varies greatly according to the species and the mode of reproduction.

**Linkage drag** The negative phenotypic effects (e.g., lower yield) on the recurrent parent associated with the loci of the donor parent tightly linked to the locus of interest being backcrossed.

**Logarithm of the odds ratio (LOD)** A logarithmic value (base 10) of the ratio between the probability of the presence of a QTL vs. its absence. A LOD value of 3.0 indicates that the probability of the presence of the QTL is 1,000-fold higher than its absence.

**Metanalysis** A comprehensive analysis based on the data of several mapping populations of the same species. The objective is to obtain a better resolution of the LOD profile of the QTLs for the traits of interest.

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**Near isogenic lines (NILs)** A set of two or more inbred lines that share most of the genome except for a small portion that contains functionally different alleles at the target locus. NILs are commonly used for the positional cloning of a locus of interest.

**Phenotypic selection** Selection based on the observation of the phenotype at different levels of functional organization based on the target trait(s). If the selected trait is highly influenced by environmental conditions and has low heritability, the effectiveness of phenotypic selection quickly decreases.

**Pleiotropy** Condition where a single locus controls more than one trait. It is more common for biochemical traits.

**Positional cloning** A series of procedures to clone a locus of interest. Positional cloning is based on the joint analysis of phenotypic data and genotyping profiles of near isogenic material with recombination events at the target region.

**Quantitative trait locus** A portion of DNA that influences the expression of a quantitative trait. The presence of QTLs is determined through appropriate statistical analysis of phenotypic and molecular data of a mapping population (e.g., linkage mapping) or a collection of unrelated genotypes (e.g., association mapping).

**Recombinant inbred lines** A collection of homozygous lines (usually from 150 up to 400) obtained following subsequent selfings (usually four or five) of an equivalent number of randomly chosen $F_2$ plants.

**Reverse genetics** An approach for discovering the function of a locus by analyzing the phenotypic effects of specific sequences obtained by DNA sequencing. Reverse genetics attempts to connect a given genetic sequence with specific effects on the organism.

**Synteny** The physical colocalization of linked loci on the same chromosome among different species. Study of synteny can show how the genome of phylogenetically related species has evolved from a common ancestor (e.g., rice for cereals) through rearrangements of the genome (e.g., translocations, inversions, duplications, etc.) in the course of evolution.

## Definition of the Subject

Attaining global food security by means of increased crop productivity will require an increase in gains from selection achieved through conventional breeding. To this end, the identification of molecular markers associated with loci controlling traits of agronomic interest coupled with the exploitation of marker-assisted breeding (MAB) approaches provides the opportunity to accelerate gain from selection. In particular, marker-assisted selection (MAS) and marker-assisted backcrossing have been widely adopted to improve resistance to diseases and other relatively simple traits. Notwithstanding these remarkable achievements, the improvement of yield and other complex quantitative traits via MAB has been marginal, mainly due to the difficulty in identifying major quantitative trait loci (QTLs) with an adequately stable effect across environments and genetic backgrounds. Additionally, the effect of most QTLs affecting yield is too small to be detected with either biparental mapping or association mapping. Genomic selection (GS) circumvents this problem by using an index for the selection of unmapped QTLs of small individual effects but with otherwise sizable effect at the whole plant level when selected together. GS is already having a positive impact on the improvement of crop yield, mainly in the private sector where high-throughput infrastructures allow breeders to handle the large number of molecular datapoints that are required for effectively deploying GS. Ultimately, an effective exploitation of MAB to enhance crop performance will rely on a closer integration between molecular approaches and conventional breeding.

## Introduction: Global Food Security and Plant Genomics

During the past century, plant breeders have been very successful in constantly raising crop yields to a level sufficient to meet the global demand in food, feed, and fiber. For wheat and rice, the two most important staples of humankind, the so-called Green Revolution spearheaded by Norman Borlaug, awarded the Nobel Peace Prize in 1970, provides the most spectacular example of the contribution of science toward an improved food security [1, 2]. Similar progress has been achieved also in maize, particularly following the

introduction of hybrids [3]. This notwithstanding, during the past decade, the rate of increase in yield in cereals, especially wheat and rice, has not met the global demand [4] as shown by the substantial decrease in the amount of global cereal reserves. Additionally, during the past two decades the number of chronically hungry people has increased and is fast approaching one billion. A number of reasons have contributed to this worrisome scenario that has already sparked food riots (e.g., during the 2007–2008 food crisis and also in 2009) and social unrest in a number of less-developed countries. An even bleaker picture looms on the horizon, when mankind will reach a projected nine billion in 2050. Consequently, an acceleration in the rate of gain in crop yields is a must in order to keep up with the need of a burgeoning population that increasingly seeks a protein-enriched, nutritionally balanced diet. The challenge faced by modern breeders is even more daunting in view of (1) global warming and the consequent increased frequency of drought, floods, high temperatures, etc., (2) the decreased availability of natural resources (e.g., water, fertilizers, arable land, etc.), (3) the increasing cost of fuels, (4) the necessity to safeguard the remaining biodiversity, and (5) the increased societal awareness of the critical need to improve the long-term sustainability of agricultural practices and decrease its impact on the environment. More simply, agriculture will need to produce more with fewer resources and more sustainably.

In this daunting scenario, genomics has ushered in a new breeding paradigm based on molecular approaches and platforms that in some cases have already contributed to accelerate the yield gain commonly achieved through conventional breeding practices [5–13]. However, a more widespread adoption of genomics-assisted selection will require the definition of new strategies based on a more effective integration of conventional and nonconventional breeding approaches as well as agronomic practices [14]. Clearly, a better knowledge of the genetic factors that determine yield and its variability from season to season will be instrumental in devising effective marker-assisted breeding (MAB) strategies for enhancing crop performance under a broad range of environmental conditions. As compared to conventional breeding approaches, MAB approaches offer unprecedented opportunities to dissect the genetic control of traits, particularly those that are quantitatively inherited, such as biomass production, yield, and many other agronomic traits selected by breeders.

## Molecular Dissection of the Genetic Control of Traits Governing Crop Performance

The first step for the dissection of the genetic control of traits that govern crop performance is the assembly of a linkage (genetic) map based upon the data of the molecular profiles of the marker loci – from as few as 100 up to several thousand – surveyed in a mapping population, usually comprised of ca. 150–200 genotypes such as $F_2$ plants, $F_3$ families, recombinant inbred lines (RILs), doubled haploids (DHs), etc., usually derived from the cross of two parental lines differing for the trait(s) of interest. The assembly of a genetic map is based on the level of linkage disequilibrium (LD, i.e., the level of nonrandom assortment of alleles at different loci) among adjacent marker loci on the same chromosome. Accordingly, mapping the loci that control the target trait is also based on the LD between the locus and nearby markers.

The estimated genetic distance between loci (markers or genes) is a function of the average number of recombination events (i.e., crossing-overs) between them at meiosis. The measuring unit used for expressing the distances among loci along a genetic map is the centimorgan (cM), which defines the interval along which one recombination event is expected to occur per 100 gametes produced at each meiotic cycle (i.e., at each sexually reproduced generation). Because a density of one marker per ca. 10–15 cM is usually sufficient to detect the presence of a functionally polymorphic locus with a major effect on the phenotypic variability of a mapping population, the number of well-spaced markers required to adequately sample the targeted species varies from as little as 100–120 as in the case of rice – one of the crops with the smallest genome size (ca. 0.45 billion bp) – to well over 300 for large genomes such as in bread wheat (ca. 16 billion bp). The desired level of genetic resolution will depend on the objective being pursued and the type of genetic materials being used.

For breeding purposes, a density of one marker every 5–10 cM is sufficient for most applications when dealing with elite cultivars. Nonetheless, for the

introgression of a particular gene (e.g., a locus for disease resistance) from a wild relative of the crop to the crop itself, a high resolution is desirable in order to avoid the negative effects of the so-called linkage drag caused by negative effects of wild alleles at the loci closely linked to the one being targeted for introgression. A much higher genetic resolution is required when the goal is the cloning of the sequence that affects the target trait. In this case, the screening of several thousands of individuals is required to reach the desired level of resolution.

Cloning the loci that govern a particular trait can be achieved via either forward- or reverse-genetics approaches, or their combination. While forward genetics focuses on the phenotype as starting point, reverse-genetics approaches rely on sequence and functional information of candidate sequences (e.g., expressed sequence tags: ESTs) that are postulated to play a role in the expression of the target trait [15]. Although most results in the dissection of the genetic basis of crop performance and agronomic traits have been obtained via forward genetics, the use of reverse-genetics approaches in *Arabidopsis* and other model species (e.g., resurrection plants, rice, *Brachypodium*, etc.) has been instrumental to elucidate the genetic networks of the signaling pathways that regulate the adaptive response of plants to abiotic and biotic constraints [16–18]. Notably, the spectacular decrease in sequencing costs [19] and the increased availability of sequence information in public databases make the reverse-genetics approach increasingly attractive and feasible.

Following the assembly of the first genetic maps based on the molecular profiling of RFLPs (restriction fragment length polymorphisms; [20, 21]), the introduction of AFLPs (amplified fragment length polymorphisms; [22]), SSRs (simple sequence repeats; [23]), and DArT (diversity array technology; [24]) markers improved substantially the assembly of genetic maps. More recently, high-throughput platforms based on SNPs (single nucleotide polymorphisms), the most frequent polymorphism in living organisms, have enabled a quantum leap in saturating maps with thousands of markers [25–29]. Notably, the spectacular advances obtained with next-generation sequencing (NGS) technology will soon allow for the resequencing of entire mapping populations and association mapping panels of species for which a template sequence is available, thus providing an almost endless supply of markers [30–34].

Once all the molecular and phenotypic data are available, statistical tests will be applied to verify whether the means of the trait values of the genotypes carrying different alleles at a particular marker are significantly different. A test statistic larger than a threshold value rejects the "null hypothesis" (i.e., the mean is independent of the genotype at a specific marker locus) and implies a significant association between the investigated marker and a linked locus that affects the phenotypic value of the target trait. The exploitation of syntenic relationships among phylogenetically related crops has greatly contributed to the identification of additional markers at target regions [35–37] and, most importantly, candidates for the investigated traits, particularly when the genome sequence of one or more of the syntenic species becomes available. This is the case of cereals, where the annotated sequence for rice, *Brachypodium*, sorghum, and maize has allowed for the identification of conserved orthologous set (COS) markers from ESTs that have maintained their microlinearity throughout evolution and speciation [37]. These markers are particularly valuable to assess the possible role of candidate genes in species not yet sequenced (e.g., wheat) and to identify orthologous sequences that have maintained their functions and colinearity across species. Thus, a good understanding of the syntenic relationships at regions underlining a QTL for rather simple traits can provide excellent clues to pinpoint the most likely candidate.

Notably, mapping loci controlling the target traits allows breeders to implement marker-assisted selection (MAS) on the basis of the polymorphic molecular markers flanking the relevant loci. Traits are usually categorized as monogenic (qualitative or Mendelian traits controlled by a single locus) and polygenic (or quantitative; controlled by many loci), the latter being highly influenced by environmental conditions and considerably more difficult to improve consequent to their lower heritability, [38]. Quantitative traits (e.g., flowering time, plant height, biomass production, yield, etc.) are particularly important for breeding purposes. Although the genetic dissection of both qualitative and quantitative traits relies on similar principles, the latter requires more extensive phenotyping and much larger mapping populations.

The prevailing assumption in the field of quantitative genetics has been that continuous variation in trait performance is caused by the segregation and action of multiple genes with a rather similar effect on the phenotype, together with a major influence of the environment which acts like some sort of "statistical fog" that blurs and limits our capacity to identify the genes that control the target trait. These genes, also referred to as polygenes, are known as quantitative trait loci (QTLs; [39]). Although the original concept – but not the acronym – of QTL mapping was first suggested in 1923 [40], the dissection of quantitative traits became eventually possible in the 1980s and the 1990s with the introduction of molecular marker platforms that allowed for genome profiling with the needed level of genetic resolution [41–45]. Two decades of dedicated experiments indicate that most QTL effects are of small magnitude as originally predicted by the so-called infinitesimal model [38, 46, 47]. This notwithstanding, a limited number of so-called major QTLs have shown a rather large effect and, in a number of cases, have been cloned [48, 49]. Once a QTL has been cloned, both genomics and genetic engineering offer additional opportunities for tailoring improved cultivars and crossing reproductive barriers among species, thus expanding the repertoire of genes available to breeders. In view of the importance of quantitative traits in breeding activities and crop performance, particular attention should be devoted to QTL mapping and the implementation of MAB for this category of traits.

### Biparental Linkage Mapping

The early studies in QTL mapping were conducted based on the analysis of the means at single markers using simple test statistics, such as linear regression, *t*-test, and analysis of variance. Because a genome-wide survey typically involves a large number of markers, the probability of detecting one or more false positives at the whole-genome level quickly increases unless the threshold of significance is adequately readjusted according to the number of tested markers [50]. Typically, a threshold level of $P_{0.05}$ entails a false-positive discovery rate (i.e., declaring the presence of a locus able to affect the target trait when actually there is no locus) of approximately 5%. Consequently, a mapping experiment based on 100 markers tested at $P_{0.05}$ will identify, on average, five markers putatively associated with loci even when no real locus segregates in the population. In order to avoid this problem, the significance threshold is corrected accordingly through a multiple test adjustment (e.g., Bonferroni's or Tukey's) that will adjust the *P* level according to the number of independent statistical tests that are performed. This notwithstanding, a much more critical shortcoming of this single-marker approach is that no information is provided on the most likely position of the locus and its effects on the phenotype. Due to these major limitations, single-marker analysis was quickly replaced by interval mapping and similar methods based on the estimated linear order of markers on a genetic map. In comparison to single-marker analysis, interval mapping provides a much more accurate estimate of the position and genetic effects of each locus [51–53]. In interval mapping, statistical methods are applied to test for the likelihood of the presence of a QTL. The result of the likelihood tests carried out at regular intervals across the ordered markers is expressed as LOD (Logarithm of the ODds ratio) scores, computed as the $\log_{10}$ of the ratio between the chance of a real QTL being present given the phenotypic effect measured at that position, divided by the chance of having a similar effect when no QTL is present. Thus, LOD values of 2.0 and 3.0 indicate that the presence of the QTL is 100- and 1,000-fold more likely than its absence, respectively. The graphical output is an LOD profile that allows one to compute an empirical confidence interval (usually computed as LOD – 1) around the QTL peak. In order to avoid declaring false-positive QTLs (i.e., declaring the presence of a QTL when the QTL is actually absent), a reasonably high threshold value for the LOD score should be set (usually $> 2.5$). Iterative software based upon resampling procedures provides a more accurate estimate of threshold values according to the size of the mapping population and the number of markers [54].

Epistasis can greatly influence the outcome of interval mapping. This problem can be partially overcome with the use of composite interval mapping, a statistical procedure that can account for the effects of other QTLs inherited independently from the interval (i.e., chromosome region) being considered, thus reducing the possibility of detecting "ghost" (i.e., false) QTLs. Compared to single-QTL interval mapping, statistical

approaches for locating multiple QTLs are more powerful because they can differentiate between linked and/or interacting QTLs that will otherwise go undetected when using single QTL interval mapping. Given the potential impact of epistasis on the response to selection, quantifying its influence on target traits is an important component for designing and organizing any MAS strategy [55]. It is likely that the incorporation of epistatic interactions into more properly devised statistical models will play a relevant role in explaining complex regulatory networks governing the expression of quantitative traits.

A major shortcoming of QTL studies is the low accuracy in detecting the real number of QTLs affecting the genetic variation of the investigated traits, particularly with populations of less than 150–200 families, which is the case in the majority of QTL studies reported so far. A simulation study applied to experimental data showed that with populations of ca. 100–200 families only a modest fraction of QTLs was identified; furthermore, the effect of each single QTL was usually overestimated [56]. Another study showed that detection of QTLs of small effect is very difficult with mapping populations with less than 500 families [44]. These predictions were supported in experiments carried out with maize mapping populations large enough (>400 families) to allow for a meaningful subsampling [57, 58]. Therefore, the chance of detecting a QTL in several environments is small even in the absence of QTL × Environment (QTL × E) interaction. Accordingly, inconsistency of QTL detection across environments has been repeatedly reported [59, 60].

### Association Mapping

In the past decade, as an alternative to linkage mapping with biparental populations, association mapping based on the evaluation of panels of unrelated accessions (ca. 150 or more) has been adopted as an additional option for trait dissection [61–65]. The assumption underlying the use of association mapping to detect the presence of loci influencing the target trait is that alleles at two closely linked loci share a historical ancestor, and this original co-occurrence will gradually decay in the population due to recombination events during subsequent meioses. Consequently, the relative allele distributions of an unknown gene and that of a closely linked marker will be nonrandom because the two are in LD. A major factor to be considered for a correct application of association mapping is the presence of population structure, which will significantly bias the results and inflate spurious marker-trait associations (i.e., declaring false positives). Algorithms and methods are being developed to correct for these effects. An important advantage of association mapping is that the linkage is evaluated over the large number of historic meiosis, which in turn entails a much lower LD and higher genetic resolution as compared to linkage mapping with biparental populations. Another advantage is that the genetic variability explored by a large panel of unrelated accessions is much larger than that present in a segregating population derived from two parental lines. Conversely, a major shortcoming of association mapping is that it does not allow for the detection of the effect that a rare, but otherwise agronomically valuable, allele may have on the target trait. In fact, the statistical procedures used for revealing the effects associated to a particular locus/haplotype consider only alleles with a frequency higher than 10% over the entire population; alleles with a frequency lower than 10% are considered rare and as such, are discarded. The cutoff threshold of 10% has been introduced to reduce the ascertainment bias that a small sample (i.e., less than 10%) of accessions would inevitably introduce, being unable to correctly represent the effect of that particular allele at the level of the entire population [62]. Clearly, this is not an issue when dealing with mapping populations where allelic frequencies are expected to be equal to ca. 50%, barring the presence of genetic factors that might influence the transmission of gametes carrying the different parental alleles. In association mapping, the procedure of discarding the individuals carrying rare alleles inevitably reduces the statistical power to identify the role of such loci in controlling the variability measured for the target trait. An example of this has recently been reported in durum wheat, where a locus with a large effect on yield in a biparental cross [162] showed no appreciable effect in a parallel association mapping study where only one of the parental alleles was considered, due to the fact that the other parental allele was present in low frequency [65].

The main factors to be carefully considered for optimizing the effectiveness of association mapping are the level of LD among the investigated accessions and the presence of population structure that could greatly increase the false-discovery rate (i.e., type-I error). Closely related to the concept of LD is the concept of "haplotype," which can be defined as the chromosome fragment carrying a common set of marker alleles in close linkage at adjacent loci [66]. When using haplotypes in association studies, the information of several linked bi-allelic markers is combined as a single, multi-locus informative marker. Haplotypes can be generated *in silico* from sequences deposited in the database, by resequencing target loci (sequence haplotypes) or genetic maps (marker haplotypes). Therefore, haplotypes will extend according to the level of LD, the value of which varies greatly (up to 100-fold or even more) not only among species, but also within a single species according to the frequency of crossing-over events in each chromosome region. As an example, centromeric regions are characterized by very low recombination if compared to subtelomeric, gene-rich regions. Populations characterized by high LD (i.e., extending for $>$ 1 cM, corresponding to several million base pairs (bp) depending on the ratio of the genetic and physical distance) are best suited for a genome-wide search [65]. Alternatively, the utilization of panels with a low LD (i.e., extending $<$ 10,000 bp, typically a small fraction of 1 cM), a condition that is typical of allogamous species like maize [67], allows for a much higher level of genetic resolution and for the validation of a candidate sequence. Clearly, the level of LD influences the number of markers/cM required to obtain meaningful information. As compared to a low LD condition, a high LD level is associated with a proportionally longer haplotype, hence requiring a lower number of markers to conduct meaningful genome-wide surveys. This feature is more prominent in elite materials that have undergone high selection pressure as a result of modern breeding practices, which in most cases has led to a reduction of haplotype diversity as compared to locally grown landraces and, more notably, wild relatives of crops that have not gone through the domestication bottleneck. As an example, LD in wheat – a selfing species that has undergone a very stringent selection mostly due to the importance of quality parameters required by the food industry – extends up to 5–10 cM [65], while in outcrossing species like maize LD is usually below a fraction of cM or even less than 10,000 bp [68]. An example of the high level of genetic resolution made possible through association mapping is shown by the fine mapping and, in one case, positional cloning of QTLs for flowering time in maize [67, 68]. In particular, association mapping revealed that the most important QTL for flowering time per se (i.e., independently from photoperiod sensitivity) in maize corresponds to a 2.3 kb, noncoding, long-distance enhancer region located 70 kb upstream of a gene known to regulate flowering time also in *Arabidopsis* [49]. Another remarkable example in which the functional polymorphism responsible for phenotypic variability was assigned to a noncoding region far (ca. 5,000 bp) from the structural gene has been reported in sorghum through the cloning of a major QTL for aluminum tolerance [69]. Clearly, only a positional cloning approach is able to unequivocally highlight the role of noncoding regions in controlling the level of expression of a particular gene and the resulting phenotype. To what extent noncoding, long-distance enhancers might be involved in regulating the expression of quantitative traits is presently unknown. Notwithstanding the importance of this issue for a more complete understanding of the regulation of gene expression, this level of genetic dissection is certainly not required from a breeding standpoint, since both MAS and genetic engineering would still allow breeders to fully exploit the beneficial effects linked to either natural allelic variation or the ectopic expression of the structural locus encoding for the target trait.

Despite the clear advantages of association mapping on biparental linkage mapping (e.g., multiallelism, higher genetic variability and genetic resolution, no need to assemble a mapping population, shorter time required to identify relevant loci, etc.), a major limitation of the former is represented by the high rate of false positives (i.e., Type-I error rate), hence spurious association, due to the presence of hidden population structure among the accessions being evaluated [62]. An additional constraint to a more widespread utilization of association mapping for the dissection of physiologically complex traits may derive from factors other than statistical issues.

For highly integrative and functionally complex traits such as yield, particularly under adverse conditions, association mapping may quickly lose its effectiveness as the level of functional complexity of the target trait increases. In this case, similar phenotypic values in different genotypes can result from the action of different gene networks and/or trait compensation (e.g., yield components), thus undermining the identification of significant marker-trait association across a broad range of genotypes such are those usually present in the panels used for association mapping. Although a similar limitation also pertains to a mapping population developed from the cross of two divergent lines, its relevance in the case of association mapping for complex traits is greatly increased by the much wider functional variability explored with association mapping. This is particularly the case whenever the investigated trait (e.g., yield under drought conditions) is strongly influenced by differences in phenology, mainly flowering time and/or plant height; in this case, the overwhelming effects on yield of phenological traits will inevitably overshadow the effects due to the action of loci controlling yield per se, i.e., irrespectively of flowering time and plant height.

### Comparative QTL Mapping and Metanalysis

A major shortcoming in QTL mapping is the limited accuracy in identifying the most likely position of each single QTL on the chromosome. Unless highly isogenic materials are evaluated, the confidence interval in assigning a QTL is rarely shorter than 10 cM, an interval likely to contain several hundred genes. The availability of QTL data for two or more mapping populations of the same species allows for the comparison of the position of QTLs by means of a metanalysis carried out with dedicated software [70]. This, in turn, provides a better genetic resolution of the QTL interval and reduces the confidence interval around the peak of the LOD profile. This exercise is particularly useful when a reference map with hundreds of well-spaced markers is available and contains "anchor markers" (usually RFLPs, SSRs, and/or SNPs) also used to investigate other mapping populations of the same species. An additional advantage of a reference map is that it allows one to compare the map position of QTLs with that of mutants for the same trait, thus contributing relevant information for the identification of possible candidate genes causally affecting the investigated trait. Accordingly, Robertson [71] suggested that a mutant phenotype may be caused by an allele with a much more drastic effect in comparison to that of QTL alleles at the same locus, a hypothesis that has been validated in maize for a QTL for plant height colocalized with the mutant *dwarf3* [72]. These results indicate that no real boundary exists between Mendelian and quantitative genetics, while suggesting that loci can be classified in either category based upon the magnitude and heritability of the effect of the alleles being considered. It follows that the information provided by mutants is of great value for QTL studies and breeding applications.

### Isogenic Materials for Mapping and Cloning QTLs

A valuable opportunity for investigating the effects of a particular QTL and eventually isolate the functionally polymorphic sequence responsible for its effects is offered by the analysis of pairs of isogenic materials (e.g., near isogenic lines: NILs) contrasted for the parental chromosome regions (usually ca. 10–30 cM long) present at the target QTL. NILs can be obtained through repeated selfings of $F_3$-$F_5$ individuals heterozygous at the QTL region prior to isolating the homozygotes for each one of the two parental segments carrying the functionally contrasting QTL alleles [73]. Alternatively, each parental line of the mapping population originally evaluated for discovering the QTL can be used as recurrent parent in a backcross scheme in which a single genotype heterozygous at the QTL in question is utilized as donor of the alternative QTL alleles; in this case, the congenic lines are identified as backcrossed-derived lines [74]. With NILs, it is thus possible to "mendelize" major QTLs characterized by a sizable additive effect. Unlike genome-wide QTL studies where more than 100–150 genotypes are usually screened, experiments conducted with NILs involve few genotypes (two as a minimum), thus allowing for a much more refined and detailed phenotypic evaluation of the effects of the QTL [74, 75]. However, it should always be appreciated that the results of NIL-based studies could to a certain extent be biased by the action of one or more closely linked genes affecting the investigated traits, a particularly likely event when the region flanking the QTL extends for several cM.

A more systematic search of QTLs is made possible with the use of a series of isogenic lines obtained through the introgression, via backcrossing, of a small portion (ca. 20–30 cM) of the genome of a donor line into a common recurrent line, usually an elite cultivar [76]. The final objective is to assemble a collection of so-called introgression library lines (ILLs; at least 70–80 or more lines for each cross), basically a collection of NILs, each one differing for the introgressed chromosome portion and collectively representing the entire donor genome [76]. A major advantage of ILLs is the rapid progress that they allow for the fine mapping and positional cloning of major QTLs [48, 77]. Besides the well-documented effectiveness of ILLs for the mapping and cloning of QTLs in tomato [77, 78], ILLs have been instrumental for mapping drought-adaptive QTLs in rice [79] and maize [163]. Once ILLs are made available and major loci for the target traits are identified, testing for epistasis becomes particularly feasible using a small number of genotypes, unlike with mapping populations, where an accurate testing for epistasis will require the evaluation of at least 200 families.

The availability of NILs for a major QTL is an important prerequisite for undertaking the cloning of the sequence underlying the trait being targeted. Besides contributing to a better understanding of the functional basis of quantitative traits [68, 80], QTL cloning provides an essential opportunity for more effectively mining and exploiting the allelic diversity present in germplasm collections [49, 82]. Recent advances in high-throughput profiling and sequencing of both the genome and transcriptome coupled with reverse-genetics approaches/platforms (e.g., collections of knockout mutants, TILLING, RNAi, etc.) have streamlined the procedures and markedly reduced the time required to identify the sequences governing variation in quantitative traits. Until now, the molecular dissection of a candidate locus has been prevailingly achieved through positional cloning and association mapping. Both approaches exploit LD to identify the most promising candidate gene(s) and benefit from the map information of candidate genes and mutants in the species under investigation and in closely related ones. As sequence information accumulates and our understanding of biochemical pathways improves, QTL cloning via the candidate-gene approach becomes an attractive alternative to positional cloning, particularly for traits underlined by a known metabolic pathway [83, 84].

## Modeling QTL Effects

QTL-based modeling holds promise to allow for a more effective design of "molecular ideotypes" on the basis of estimated QTL effects for growth parameters of response curves to environmental factors revealed by exposing mapping populations to such environmental factors [85–87]. Additionally, crop modeling provides useful clues to unravel the genetic basis of G × E interactions and toward a better understanding of traits' plasticity [88], a feature of increasing importance in view of the effects on crop growth and yield due to the enhanced vagaries in weather conditions consequent to global warming. An accurate estimate of the consistency of QTL effects in a particular genetic background can be obtained through extensive testing of the genetic materials under different environmental conditions as to level of irrigation, nutrients, temperature, etc.

In maize, an ecophysiological model and QTL analysis have been integrated to investigate the genetic basis of leaf growth in response to drought and predict leaf elongation rate as a function of estimated QTL effects at varying air humidity, temperature, and soil water status (Tardieu 2003). QTLs with a limited QTL × E interaction and with a linear response to a particular environmental factor will provide more predictable opportunities to improve crops' performance through MAS. An important issue rarely addressed in view of the inherent difficulty in doing so from an experimental standpoint under field conditions is that crop performance is often constrained by more than one environmental factor (e.g., drought and heat) occurring simultaneously, a condition which greatly undermines the prediction of QTL effects, particularly when considering multiple QTLs.

## Marker-Assisted Breeding to Improve Crop Performance

The improvement of crop performance through conventional breeding has for the most part been achieved with little or no knowledge of the genetic basis of the selected traits, particularly yield and its underlying

morphophysiological determinants. The main obstacle to raising crop yield via conventional breeding by means of phenotypic selection is represented by the low heritability of yield, particularly under marginal conditions and low-input agriculture (e.g., low supply of nutrients and/or water). As an alternative to phenotypic selection, MAB can be applied to more effectively improve crop performance. The ultimate goal of MAB is to increase the cost-effectiveness of the selection gain per unit time. Although the costs entailed by MAB are still quite high when compared to conventional breeding practices, the sizable reduction in the time required to release an improved cultivar made possible through MAB can justify its application once agronomically valuable alleles at target loci (genes or QTLs) are identified. The convenience of adopting MAB to improve the efficiency of the selection process should be carefully evaluated on a case-by-case basis. The success of MAB will depend on the identification of the agronomically beneficial alleles at target loci, their effect in the different elite genetic backgrounds prevalently grown by farmers and their pyramiding in the correct combinations. MAB could thus be regarded as an extension and evolution of the so-called ideotype breeding, an approach based on phenotypic selection for an ideotype characterized by those morphophysiological features deemed necessary to maximize yield. As compared to ideotype breeding, MAB allows us to dissect the genetic basis of key traits and to piece back together the best alleles in a sort of molecular jigsaw puzzle, the main limitation being that only a very small number of the jigsaw tassels (i.e., genes and QTLs) have been identified. This approach, referred to as "breeding by design" [89], extends the concept of "graphical genotypes" first introduced by Young and Tanksley [90] to portray the parental origin and allelic contribution of each genotype on a genome-wide basis. Although a breeding-by-design approach is technically applicable to all major crops, its impact has been much more tangible for traits with a simple genetic control (e.g., quality, disease resistance; [91–95]) as compared to more complex quantitative traits, such as yield under adverse environmental conditions [60], a result mainly due to our rudimental understanding of the genetic basis of the latter category of traits, their interaction with environmental factors and, most importantly, the difficulty in predicting the phenotypic value of a new genotype tailored through MAB for several QTLs. Along this line, it should be underlined that the effects of QTL alleles for complex traits (e.g., yield) characterized by a large G × E interaction can drastically change according to the conditions (e.g., water availability along the crop life cycle) present in the environment being targeted.

The molecular profiles obtained with molecular markers provide the basic information required to identify the haplotype of each individual plant at a target locus. Haplotype profiling of collections of elite cultivars released during the past decades and derived from a limited number of founders (i.e., genotypes that in view of their positive features have been frequently used by breeders as parental lines) provides a means to identify the chromosome regions that have been preferentially retained throughout the breeding activities carried out during such time period. It is plausible to hypothesize that these chromosomal regions harbor loci (genes or QTLs) important for the selection of improved cultivars.

The strategies deployed to improve crop performance based on molecular information can be categorized according to the level of knowledge and understanding of the loci that underline the phenotypic traits under selection. While MAS and marker-assisted recurrent selection (MARS) during the past two decades have deployed allelic variation at mapped loci often characterized by a rather large effect on the phenotype, the new paradigm ushered in by genomic selection (GS) via high-throughput profiling has emphasized the selection of unmapped, uncharacterized loci with rather limited individual effects but with otherwise sizable effects when selected together. The next sections will critically analyze some of the main features of these rather different approaches that should not be regarded as antagonistic, but rather complementary.

## Marker-Assisted Selection

Once loci are mapped and their effects characterized, the two most common applications of MAS in crop breeding are to (1) accelerate the backcross (BC) procedures required to transfer beneficial alleles at one or more loci into an elite cultivar and (2) facilitate the selection of one or more target traits within

a segregating population. The former application is the one that so far has been most frequently adopted in breeding programs and is usually referred to as marker-assisted backcross (MABC). MAS has also been deployed frequently to create isogenic lines (e.g., NILs, introgression libraries, etc.). These materials are used to identify and map genes/QTLs and, as such, usually do not impact directly on the outcome of breeding practices and the release of improved cultivars.

As compared to the conventional BC procedure, MABC based on the use of markers uniformly spaced along the genome (ca. 20–25 cM apart) can save three to four BCs in recovering most of the genome of the recurrent parent, thus reducing the time required for the release via BC of the improved version of the recurrent parent [96]. The advantage is greater for the incorporation via BC of recessive resistance genes, the phenotypic detection of which is only possible for the homozygous individuals carrying recessive alleles at both loci. In this case, phenotypic selection takes twice longer as compared to dominant alleles, since a selfing generation is required after each BC for the phenotypic identification of the homozygous recessive resistant plants to be used for the next BC. The utilization of codominant markers (e.g., SSRs) allows for the identification of heterozygous plants carrying the resistance-encoding allele directly in $F_1$, thereby saving one generation for each BC cycle. During the past two decades, MABC has been routinely deployed by seed companies to introgress beneficial alleles from unadapted accessions (e.g., landraces or wild, sexually compatible relatives of crops) and particularly to introgress transgenes into elite materials [9, 97, 98]. At each generation, individuals heterozygous at the region flanking the target locus are identified based on the results of molecular profiling. In comparison to conventional BC, MABC provides additional, distinct advantages such as (1) avoiding the vagaries in phenotyping when the conditions do not allow an accurate classification of the progeny segregating for the target trait (e.g., absence of the pathogen when backcrossing an allele for resistance to the disease), (2) reducing the number of plants to be screened in each selection cycle, and (3) identifying plants with the shortest possible chromosome segment introgressed from the donor line. The latter factor is particularly important when the donor is a wild accession of the recurrent, elite line being backcrossed. In this case, the introgressed chromosome segment flanking the target locus is likely to contain many alleles with a detrimental effect on quality and yield. Therefore, it is necessary to select individuals with the shortest possible chromosomal fragment contributed by the donor parent. An additional benefit is when the phenotyping of the trait under transfer is expensive and/or cumbersome like in the case of genes affecting tolerance to diseases/pests that require artificial inoculation in order to correctly identify those plants carrying the tolerant alleles (e.g., resistance to nematodes; [99]). Other cases where MABC provides a distinct temporal advantage as compared to conventional procedures is when the phenotypic evaluation of the target trait is destructive or when the trait is expressed after flowering. Selection before flowering greatly reduces the number of plants to be selfed or crossed, thus reducing the operating costs, particularly with species with a long life cycle.

During backcrossing, different rates of recovery of the recipient genome are expected at the target region and the nontarget chromosomes. Because each BC reduces by half the percentage of the donor genome at nontarget regions, at least six or seven BCs are required for a satisfactory recovery (ca. 99%) of the recipient genome. However, the number of BCs is frequently higher due to residual linkage drag around the target locus and it is not uncommon that up to nine or ten BCs are implemented before the improved cultivar is finally released. Clearly, the longer the time required to complete the BC procedures, the lower the probability of success of the new cultivar, since other improved, competing cultivars will be released in the meantime. Simulation and practice have both shown that in a moderately sized population of a species with a relatively small genome (<500 million bp, such as rice) using more than two to three well-spaced markers per chromosome arm hardly brings any additional benefit. For a species with large chromosomes (e.g., wheat, ca. 16 billion bp), a larger number of markers in each chromosome are beneficial. With an increasing genome size, more independent recombination events are needed to reduce the contribution of the donor parent, which in turn requires a larger population size. To what extent the contribution of the donor

parent should be reduced will depend on the type of alleles carried by such fragments and, most importantly, the genetic distance between the donor parent and the recurrent parent. Nowadays, the availability of large number of SNPs in most of the major crops facilitates the screening of the BC individuals to verify in great detail to what extent the genome of the donor parent has been retained.

Formulas are available to compute the level of concordance between the allelic state at the target locus and the flanking markers during the BC procedures [81]. These formulas values indicate that the level of control made possible with only one marker is insufficient to keep the risk of losing the target allele below 5% throughout five cycles of BC. Conversely, the level of control possible with two flanking markers is considerably higher even when the markers are not tightly linked to the target locus. If the BC procedure targets a QTL instead of a Mendelian locus, the uncertainty about the exact position of the sequence underlining the QTL introduces further complexity. Because the quantity of donor genes on the carrier chromosomes decreases much more slowly in comparison to the noncarrier chromosomes, after six BCs the majority of heterozygous loci with undesirable donor alleles will be on the carrier chromosome, with the vast majority included in the intact fragment flanking the target locus.

At the chromosomes not targeted by the BC procedure, it is expected that after "n" BCs, the probability that any locus remains heterozygous between the donor and the recipient is $(0.5)^n$, which means that each BC halves the residual level of heterozygosity. Consequently, six BCs ensure a level of similarity with the recurrent parent above 99%. Results in different species have shown that there may be a significant deviation from the 75% genomic portion of the recurrent parent expected in the $BC_1$ generation [100, 101], thus demonstrating the usefulness of genotype-based selection to identify plants with the highest possible portion of the genome from the recurrent parent.

### Pyramiding Beneficial Alleles at Multiple Loci

The possibility to rapidly introgress and pyramid into existing cultivars a suite of beneficial alleles allows breeders to more quickly release improved cultivars to farmers. The best examples are in the area of disease resistance. Monogenic (Mendelian) resistance based on a single major gene is usually nondurable due to the high mutation rate in plant pathogens, which can lead to the selection of new virulent strains able to overcome the physiological barrier of an individual resistance gene. Consequently, the durability of disease resistance can be increased by screening for new sources of resistance followed by marker tagging of the relevant genes and their incorporation in elite cultivars. Pyramiding identifies the procedure for stacking the beneficial resistance alleles in a single line or cultivar, which provides a more durable resistance to pathogens as compared to monogenic resistance based on a single major gene. The advantage of pyramiding multiple alleles for resistance is particularly evident with diseases that require repeated inoculation and when phenotypic selection alone is too cumbersome and fails altogether to detect and combine multiple resistance genes in a single genotype.

Direct disease screening based on phenotypic observations is not always desirable due to a number of factors: quarantine restrictions, lack of routine screening methods and informative pathogen races for discriminating specific resistance genes, host escapes, and/or the inability to identify specific genes or gene combinations due to the occurrence of race or pathogen mixtures in the field. In these cases, MAS of race-specific genes offers a viable alternative for stacking beneficial alleles in improved genotypes which will eventually turn into novel cultivars characterized by more durable resistance to rapidly changing pathogen populations. Along this line, the constant changes in pathogen populations in different environments underline the potential value of previously defeated resistance genes. In this case, MAS offers the only practical solution to maintain such genes in current cultivars since they are masked by the epistatic effects of other resistance genes that are still effective.

In all major crops, the availability of markers tightly linked to resistance loci now allows breeders to tailor new cultivars with a suite of resistance genes able to enhance durable disease resistance to highly variable pathogens [102]. In broader terms, pyramiding is also implemented for combining beneficial alleles at loci (Mendelian or QTLs) that control traits other than disease resistance. In wheat, alleles at major loci that

influence quality (e.g., semolina color, protein content, micronutrient concentration, etc.) and tolerance to abiotic stress (e.g., aluminum, boron, salinity, etc.) are routinely introgressed via MABC [94].

When multiple loci are targeted in a BC program, the minimum population size to be considered increases considerably and rapidly becomes a major limiting factor when more than three or four loci are targeted, a number that can be increased to five or six when Mendelian loci are considered. When the targeted loci are QTLs, the uncertainty of the exact location of each selected QTL adds further constraints and reduces the number of loci that can be selected with a population of manageable size. When different lines contribute the beneficial alleles, the easiest strategy is to cross them to produce recombinant progenies and select the desired individuals. Multiple crosses might be required to pyramid all the desired alleles in one single genotype. A more general framework and the underlying theory to optimize breeding schemes for gene pyramiding have been described [103].

### Marker-Assisted Selection in a Segregating Population

MAS has been extensively used for the selection of single genes conferring tolerance to diseases/pests [91, 94, 102, 104–106]. Although early simulation studies suggested the effectiveness of MAS for the improvement of biparental populations segregating for moderately complex traits [107], the first applications of MAS in maize were disappointing [57, 108]. Sweet corn is the only exception, the main reason being its much narrower genetic basis as compared to maize used for feed production, a feature that increases the reliability of predicted gains from selection and extrapolation of the effects of different loci to different populations [109]. Another feature that makes the application of MAS particularly attractive in sweet corn is the high costs associated to conventional phenotyping, in view of the large amount of grain that needs to be processed in order to obtain an accurate estimate of the phenotypic values of the progeny to be selected. MAS applications have been more widespread in the private sector as compared to public institutions, most likely owing to a lack in the latter of the infrastructure required for an effective exploitation of MAS.

Notwithstanding the remarkable progress in identifying and in some cases cloning major loci regulating agronomically valuable traits [48, 49], more limited success has been reported for MAS of quantitative traits [110], mainly due to the difficulty in identifying major QTLs with a sufficiently large and stable effect for justifying their deployment via MAS. While true QTL $\times$ E interaction due to variable expression of a trait may cause lack of consistency in QTL detection particularly with traits characterized by low to moderate heritability, the interaction between a mapping population of small size – hence with limited power in QTL detection – with variable environments is probably an equally important factor causing inconsistency in QTL detection. This is particularly evident for the improvement of crop yield under drought conditions, one of the most difficult traits to improve not only via MAS [14, 60, 111–113] but also through conventional breeding.

### Marker-Assisted Recurrent Selection

Although marker-assisted recurrent selection (MARS) was first proposed in the early 1990s [114], only recently its adoption has provided a tangible contribution to crop improvement, mostly due the difficulty in identifying multiple loci characterized by limited G $\times$ E interaction and reasonably consistent effects in different genetic backgrounds other than that in which they were originally identified. The goal of MARS is pretty much similar to that pursued in pyramiding alleles at multiple loci, i.e., accumulating the beneficial alleles at as many as possible, preferably all, loci being targeted. Pyramiding alleles at many loci (e.g., $>10$) is best achieved with a recurrent selection strategy [115]. In this case, simulation showed that with 50 QTLs and a population of 200 plants the frequency of favorable alleles reached 100% after ten cycles when markers cosegregated with the QTL (i.e., they coincided), but only 92% when the marker-QTL interval was equal to 5 cM, hence increasing the possibility of losing the desired QTL allele due to recombination. In practice, with a higher number of loci under selection the occurrence of plants carrying the desired ideal combination becomes increasingly unlikely and basically impossible when more than 20 loci are targeted simultaneously. This problem can be partially mitigated through

successive cycles of crossing individuals carrying complementary combinations of the desired alleles [89]. This concept can be extrapolated to crosses with multiple parents.

MARS can start irrespectively of knowing the map position of the desired loci, which instead can be identified during the selection process. Simulation has clearly shown the superiority of MARS over phenotypic selection (from 5% to 20%), particularly when the selected population is highly heterozygous [116]. In maize, MARS has been applied rather extensively for improving relatively complex traits such as disease resistance, tolerance to abiotic stress, and also grain yield [111, 117–119].

The outcome of both MAS and MARS within a segregating population can be influenced by the genetic makeup of the targeted genetic background in terms of alleles present at other loci that interact epistatically with the target locus, an aspect which becomes particularly relevant for quantitative traits in view of the high number of loci involved in their control. Accordingly, since most evaluations of QTL effects and MAS strategies assume that QTLs act independently [55], it has been argued that MAS has little if any power over traditional phenotypic selection [46]. With maize as a model species, computer simulation showed that gene information is most useful in selection when few loci (<10) control the trait, while with many loci (>50) the least squares estimates of gene effects become imprecise. Based on these results, the typical reductionist approach pursued through QTL discovery strongly limits the outcome of MAS carried out for traits controlled by many QTLs [46].

### Genomic Selection

In genomic selection (GS), genetic markers in number sufficient to cover the entire genome according to the level of LD are used so that most QTLs controlling the trait being selected are in LD with at least one neighboring marker. Unlike in MAS, in GS the individual plants are chosen without mapping the underlying QTLs that remain unknown along the entire process. Originally devised for animal breeding, only recently has GS been adopted for improving crop performance [120–122]. This was due to the fact that only in the past few years its application has become technically feasible in plants thanks to the introduction of SNP profiling with a level of genome saturation sufficient to detect the cumulative effects of the plethora of minor QTLs affecting quantitative traits which, on a single basis, will inevitably remain undetected in a biparental mapping population.

In GS, the breeding values of all the markers distributed across the genome are fitted as random effects in a linear model. The trait values are then predicted as the sum of the breeding values of each individual genotype across all the profiled markers and selection is based on these genome-wide predictions. A simulation study showed that across different numbers of QTLs (from 20 to 100) and levels of heritability, the response to GS was from 18% to 43% higher as compared to MARS. The number of markers that are used to predict the breeding values usually varies from a minimum of ca. 200 up to 500. A higher number of markers are required as the functional complexity of the targeted trait increases and LD decreases. Notably, GS is most effective for complex, low-heritable traits controlled by a large number of QTLs.

Implementation of GS is already having a major impact on the improvement of yield and other complex traits, mainly in the private sector where high-throughput infrastructures and robots allow for the routine creation and handling of millions of datapoints. Clearly, GS is not antagonistic to either MAS or MARS. Rather, they should be deployed in a complementary fashion on a case-by-case basis and according to the availability of mapped major QTLs, the accurate evaluation of their effect, and the frequency of the agronomically desirable alleles in the germplasm under selection.

## Integrating Marker-Assisted Breeding in Conventional Breeding Projects

Among other factors, a broader application of MAB in conventional breeding projects will depend on the cost of molecular profiling [123, 124]. SNP markers are ideally suited for this role. In maize, the cost-effectiveness of MAS for the introgression of a single dominant allele into an elite line was compared with that of conventional breeding [125]. In this particular case, neither method showed clear superiority in terms of both cost and speed: Conventional breeding schemes

were found to be less expensive while MAS-based breeding schemes were shown to be faster. High-throughput genotyping based on the scoring of markers that do not need the use of gels [126–128] coupled with quick DNA extraction protocols are needed to streamline MAS and lower its cost.

An important factor to be carefully considered prior to embarking in any MAS activity targeting specific loci is the robustness of the marker-locus association and their genetic distance. Clearly, the level of LD of the genetic materials used to investigate the genetic makeup of the target traits plays a pivotal role in determining the level of genetic resolution. Accordingly, biparental $F_2$ populations have the maximum amount of LD, hence the lowest level of genetic resolution. Although this feature is advantageous for the initial QTL mapping studies in view of the limited number of markers that are required, it clearly limits the accuracy of MAS and usually does not allow us to resolve tightly linked QTLs from pleiotropic ones [129]. This problem can be circumvented by deploying genetic materials that capture a higher recombinational level, either historically (e.g., panels of unrelated genotypes suitable for association mapping; [67, 130]) or through subsequent random matings of the individuals of the original mapping population [131]. Increasing the genetic resolution not only enhances the reliability of MAS but also reduces the list of the possible candidates, an important prerequisite in identifying the sequence responsible for the phenotype of interest. Therefore, prior to undertaking an association mapping study, it is important to acquire a good understanding of the LD patterns in the set of genetic materials to be evaluated. In fact, LD can be caused by factors other than linkage. Spurious associations in a collection of germplasm accessions can be due to LD between unlinked genomic regions (i.e., >50 cM apart) on the same chromosome and/or between genomic regions located on different chromosomes. Dedicated softwares are available to reduce the frequency of false-positive associations due to the bias introduced by preexisting population structure.

One of the most critical steps in any breeding program is the choice of suitable parental lines to create the new segregating populations that will undergo selection. Ideally, such parental lines will contribute beneficial alleles at the loci most critical for the target traits and, more in general, crop performance and its quality. Molecular profiling can contribute in two major ways to expedite the selection process and increase the response to selection. In autogamous crops, MAS is applied to choose the parental lines that are crossed to generate new mapping populations (mostly biparental) and then to select during the subsequent generations the recombinant progeny that carry the desired alleles at the targeted loci. In wheat, MAS is being deployed in a number of breeding programs both in the public and private sectors [94]. In particular, more than 30 traits have been targeted, mainly for disease resistance, quality, and abiotic stress tolerance. In allogamous crops (e.g., maize) where the populations used to extract new parental lines routinely undergo recurrent selection, MARS can be applied at each selection cycle to increase the frequency of the beneficial alleles within the population until the best performing alleles are fixed within the population and, as such, no longer require selection. By increasing the frequency of beneficial alleles in a breeding population, the probability of recovering a genotype with the combination of desired alleles is increased. As an example, increasing the favorable allele frequency from 0.50 to 0.96 will increase the probability of recovering the ideal genotype for 20 independent regions from one in a trillion to one in five [9]. This change in allele frequency will improve the mean performance for the selected trait of the population and any line derived from it. Breeders can deploy different MARS schemes depending on the selection model and the desired genetic structure (e.g., inbreeding level) of the population obtained after MARS. The MARS schemes require optimization for best managing field and laboratory resources, hence containing the costs, as well as for expediting the selection process, hence the accumulation of favorable allele frequency. When several traits and loci are targeted simultaneously, a multiple trait index is used to combine the values of each individual trait into a single index and different weights are assigned according to the perceived importance of each trait. The output of this process is an estimated genotypic value calculated for each progeny being considered for selection. MARS can also be applied to autogamous crops (e.g., soybean) in order to enhance the performance of the breeding populations used to select improved genotypes that will hopefully outperform the existing cultivars.

As compared to conventional breeding practices, the outcome of MARS has clearly indicated its superiority for improving yield in maize, sunflower, and soybean [9]. Of utmost importance for the successful implementation of MARS is that breeders perform phenotypic selection on the lines per se that will be utilized for MARS. Additionally, phenotypic evaluation and selection among and within derived lines should continue after MARS.

Systematic profiling of parental lines is now routinely applied with a different level of genetic resolution, hence according to the level of LD of the target species. SSR profiling is rapidly being replaced by SNP profiling, much more effective than the former to define haplotype structure and much cheaper and amenable to high-throughput profiling. SNP platforms are particularly suited to the high-throughput profiling required by GS.

Once the template sequence of a crop becomes available, resequencing of lines can be used to obtain a far deeper understanding of their genomic architecture, allelic composition, and ultimate haplotype [132–134]. The spectacular reduction in cost that followed the introduction of second-generation sequencers makes resequencing of single genotypes a rather attractive and affordable option [135–137]. Additional progress in sequencing will further reduce the costs in as much direct resequencing of entire mapping populations may soon become more affordable than SNP profiling.

## Mining Beneficial Alleles in Wild Relatives of Crops

As compared to their wild counterparts, the domestication bottleneck that all crops went through coupled with the strong selection first empirically practiced by farmers and then more systematically by modern breeders have markedly reduced the level of genetic variability within cultivated species, an aspect even more relevant for traits playing a substantial role in survival under natural conditions [82]. This limitation can be overcome through the implementation of advanced backcross QTL (AB-QTL) analysis [138], an approach that allows breeders to quickly discover and exploit beneficial QTL alleles present in wild germplasm but otherwise absent from elite germplasm. The AB-QTL approach relies on the evaluation of $BC_2$ families between an elite cultivar utilized as recurrent parent and a donor accession, usually a wild species that is sexually compatible with the crop. Usually, QTL analysis is delayed until the $BC_2$ generation and after selection in $BC_1$ against features known to affect negatively yield (e.g., ear shattering in small-grain cereals). The effectiveness of the AB-QTL approach has been proven in tomato [138, 139], rice [140], and barley [141]. These results are encouraging for using AB-QTL as a germplasm enhancement strategy for identifying wild alleles capable of improving the yield of the related crop, particularly under low-input agriculture and marginal environments where wild alleles may prove more beneficial, particularly for yield per se and disease resistance. An essential prerequisite is that the introgression of such beneficial alleles should bear no negative consequences when crops are grown under more favorable and high-yielding conditions.

Wild relatives of crop species can contribute to the identification of novel alleles for agronomically relevant traits by focusing on those loci that molecular evidence indicates as having been targeted by selection during both domestication and modern breeding [142]. To this end, the comparison of the allelic diversity present in elite accessions, landraces, and the undomesticated wild relatives of each crop allows for the identification of loci devoid of genetic variation within the elite germplasm, most likely as a result of domestication and subsequent man-made selection. The underlying assumption is that the loss of genetic diversity observed from the wild parent to the cultivated crop highlights the strong man-made selection at loci that control the expression of agronomically important traits, particularly those relevant for adaptation to abiotic stress. Therefore, both this "diversity screen" approach and the AB-QTL approach allow for the identification of valuable loci which would otherwise go undetected due to a lack of allelic diversity in the cultivated gene pool. An additional advantage of the diversity screen approach is that it allows for the identification of candidate genes of potential agronomic importance even without prior knowledge of gene function.

## Leveraging the "-Omics" Platforms

During the past decade, a number of technologically sophisticated platforms have become available to

collect a large amount of data on the dynamics of the transcriptome, proteome, and metabolome. The availability of these "-omics" profiling data facilitates the identification of candidate genes and provides us with a more holistic picture of the molecular events characterizing functions at the cellular, organ, and plant levels and how these are influenced by environmental cues [84, 143–146].

Unlike from the classical QTL positional cloning approach in which an adequately large mapping population is basically "interrogated" in order to identify the genetic determinants of QTLs, the candidate-gene approach capitalizes on gathering experimental evidence to support and validate the causal role of a coding sequence (e.g., glutamine synthetase gene) in governing variation for the putative target trait (e.g., nitrogen-use efficiency). The major advantage of the candidate-gene approach is that it bypasses the tedious and expensive procedures required by positional cloning. Identifying suitable candidate genes and elucidating their function can be expedited by combining different approaches and high-throughput -omics platforms applied to target crops and/or to model species. From a technical standpoint, combining laser-capture microdissection with the -omics platforms offers an unprecedented level of functional resolution at the tissue level, down to a single-cell layer [145]. Among the different platforms available for the mass-scale profiling of the transcriptome, microarrays have been more frequently utilized to investigate the changes in gene expression, particularly in plants exposed to adverse conditions [147–150]. Nonetheless, microarray platforms are quickly being replaced by high-throughput transcriptome sequencing by means of second-generation sequencing platforms [151].

Additional information on the changes in cellular metabolism is provided by the profiling of the proteome [152] and metabolome [153, 154] that, as compared to the transcriptome, are functionally closer to the phenotype, thus reporting also on variability due to posttranscriptional and posttranslational regulation. However, it should be appreciated that both proteomics and metabolomics report changes for a rather limited portion (ca. 5%) of the expressed genes; additionally, proteomics is often unable to detect the changes in gene products (e.g., transcription factors) that despite their low level are more likely to play an important role in pivotal functions (e.g., signal transduction in response to biotic and abiotic stress) and consequently, to underline QTLs.

Metabolome profiling can also be used to identify loci regulating the level of a particular metabolite and verify its coincidence with QTLs for yield and/or genes involved in metabolic pathways. With the present technology, up to ca. 2,000 different metabolites can be profiled in a single sample [155]. In maize, QTLs for invertase activity have been identified in a population subjected to drought stress [156]. The number of QTLs for invertase activity detected under drought was more than twice the number detected under well-watered conditions, an indirect indication of the important role of this enzyme under drought conditions. One QTL common to both treatments was located near *Ivr2*, an invertase-encoding gene. The colocation reported between the activities of three enzymes (invertase, sucrose-P synthase, and ADP-glucose pyrophosphorylase) involved in sucrose and starch metabolism and a corresponding structural gene suggests its role as a candidate gene for explaining part of the variability in enzyme activity [157]. These studies indicate that invertase activity is an important limiting factor for grain yield in maize exposed to drought during the reproductive phase [158].

The candidate-gene approach is particularly effective when a clear cause-effect relationship can be unequivocally established between the gene product and the target trait. An example of this approach is the cloning of a QTL for cell-wall beta-glucans in barley grains based on a synteny analysis between barley and rice that revealed the presence in the syntenic portion of the rice genome of a cellulose synthase-like *CslF* gene that genetic engineering unequivocally showed to influence beta-glucans content in barley grains as well as in other species, including also *Arabidopsis* [83]. This notwithstanding, identifying suitable candidates for functionally complex traits such as yield and yield components is a much more daunting undertaking given the large number of genes that influence these traits.

## Future Directions

The first comprehensive report of DNA-based markers (RFLPs; [20]) in a crop species was published in 1986.

Since then, an almost countless number of studies have shed light on the genetic control of plant growth and functions, and, most importantly crop yield. One clear take-home message that has emerged from these studies is the existence of a continuum between Mendelian and quantitative traits that will eventually help in identifying the functional polymorphisms, either of genetic or epigenetic origin that underlie quantitative trait variation. In this respect, QTL cloning will become a more routine and easier practice thanks also to the massive resequencing of mutant collections. This, in turn, will facilitate the identification of the best performing QTL alleles, their pyramiding through MAS, and the identification of novel alleles via TILLING [159] or by means of site-directed mutagenesis at the key functional domains of the encoded proteins. It is under this QTL cloning paradigm that the molecular basis of quantitative traits will be dissected in order to advance our understanding of the genetic makeup of this category of traits and to more accurately tailor crop morphology and productivity with beneficial alleles.

From an applicative standpoint, although conventional selection based on phenotypic evaluation will likely remain the mainstay for most breeding programs, particularly in the public domain, MAB and its applications will increasingly be adopted and will in some cases become prevalent as compared to conventional practices. As the twenty-first century unfolds, a multitude of genomics and postgenomics platforms are at hand to expand our understanding of the genetic basis of crop performance and to improve the efficiency of selection procedures for the release of new, improved cultivars. Resequencing will revolutionize the way breeders deal with their germplasm and will provide unsurpassed opportunities for a deeper mining of allelic diversity and harnessing its full potential. Nonetheless, our understanding of the functional basis of yield and other quantitative traits is likely to remain rudimental. The elusive nature of the QTLs that govern yield and yield stability is a formidable hurdle toward a more effective selection targeting specific loci and a better understanding of quantitative traits. Notably, GS can and will be applied irrespective of our degree of understanding of the genetic architecture of quantitative traits. Importantly, MAS and GS should be considered as complementary rather than alternative approaches, the utilization of which should be determined on a case-by-case basis. Bioinformatics and user-friendly databases will play a pivotal role for handling and managing the deluge of data produced by the molecular and phenotypic platforms.

In terms of experimental materials utilized for QTL studies, a growing attention will be devoted to the exploitation of multiparental crosses and mini-core collections of germplasm accessions with varying LD levels. In the mapping populations so far utilized for QTL discovery, most QTLs go undetected owing to the small size of the population, the presence of functionally monomorphic alleles and the small effects of many of such QTLs. Along this line, nested-association mapping (NAM) populations provide an interesting option to take advantage of both biparental (linkage) mapping and association mapping [160]. On a finer scale, high-throughput proteome and metabolome profiling will accelerate the identification of the causative mechanisms contributing to adaptive responses to adverse environmental conditions (e.g., drought, flooding, heat, etc.) whose frequency and intensity are expected to increase due to global warming. Nonetheless, the deluge of information originated through the molecular approaches and the -omics platforms will not automatically translate into novel cultivars. A "systems biology"-like approach will be instrumental for optimizing the accurate integration and exploitation in breeding terms of all the -omics information.

From an applicative standpoint, accurate phenotyping often remains the main limiting factor for identifying novel loci [161]. Semiautomated, high-throughput phenotyping under both controlled conditions and in the field promises to streamline gene discovery and narrowing the genotype-phenotype gap that hampers a more widespread deployment of MAB in crop improvement [87]. Along this line, it is important to emphasize that any molecular approach aiming to discover genes/QTLs and test their effects should preferably be carried out in an experimental context whose results are as relevant as possible and readily applicable to the conditions prevailing in farmers' fields [150]. An effective exploitation of genomics approaches to enhance crop performance will depend on their integration with conventional breeding. Although it is not possible to predict to what extent and how quickly the latter will be replaced by MAB, the

future release of improved cultivars will be expedited and made more cost effective through a systematic marker-based manipulation of the loci that govern crop performance and the desired features targeted by breeders.

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# Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures

Suvi T. Häkkinen, Anneli Ritala, Heiko Rischer, K.-M. Oksman-Caldentey
VTT Technical Research Centre of Finland, Espoo, Finland

## Article Outline



## Glossary

**Bioreactor** A fermentor in which plant cell cultures can be cultivated in sterile, controlled, and contained condition for biotechnological production of cell biomass and/or particular protein or small molecule.

**Medicinal plants** Plants that are used for medicinal purposes; whole plants or specific plant organs or compounds derived thereof can be utilized.

**Metabolic engineering** A process to understand metabolic pathways; a targeted alteration of metabolic pathways with the aim of improved yield, quality, and/or spectrum of produced metabolites.

**Plant cell culture** Process where plant cells are cultivated under controlled conditions; may consist of differentiated tissues or organs (e.g., shoots, roots, embryos, stems) or undifferentiated cells (e.g., callus, suspension cultures).

**Secondary metabolites** Low molecular weight compounds with enormous chemical diversity often found in plants in small amounts essential for plants' defense system; many secondary metabolites are used as pharmaceuticals, dyes, flavors, and fragrances by humans.

**Transgene** A gene that has been transferred from one organism to another.

## Definition of the Subject

Plants are the most excellent designers and producers of a variety of small compounds that are beneficial to mankind as foods, medicines, and industrial raw materials. The use of medicinal plants for human health dates back to ancient history of mankind. The first written document of the use of medicinal plants can be found in Papyrus Ebers (1800 BC). Even if the use of certain medicinal plants was known to treat certain diseases – often using the trial-and-error approach – it is only less than 200 years ago the isolation of the first active chemical constituent (secondary metabolite) responsible for its pharmacological effect occurred. Today, many plant-derived compounds are used in pharmaceutical industry, and plants also serve as an important source for new lead compounds.

Many plants containing high-value secondary metabolites are difficult to cultivate or are becoming endangered because of the overharvesting. Furthermore, the chemical synthesis of plant-derived compounds is often not economically feasible due to their highly complex structures and the specific stereochemical requirements of the compounds. The biotechnological production of valuable secondary metabolites in plant cell or organ cultures is an attractive alternative to the extraction of whole plant material. However, the use of plant cell or organ cultures has had only limited commercial success so far. This is explained by the empirical nature of selecting high-yielding, stable cultures and the lack of understanding of how secondary metabolites are synthesized or how their synthesis is regulated.

## Introduction

It has been estimated that there are at least 400,000 higher plant species in the world of which only about 10% are characterized chemically to certain extent [1]. There is no doubt that the chemical diversity of plants is much greater than any chemical library made by humans, and thus the plant kingdom represents an enormous reservoir of pharmacologically valuable molecules waiting to be discovered. Plants are thus excellent organic chemists in nature and constantly respond to environmental changes by adjusting their capacity to produce natural products. Functional genomics may open entirely new avenues to screen

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unexplored medicinal plant species for their pharmacological value. Many pharmaceutical companies have now renewed their interest on plant-derived compounds due to too high expectations on combinatorial chemistry or computational drug design to obtain new drug leads during the past decades [2, 3].

Many secondary metabolites of industrial value are complex in their structures making chemical synthesis very challenging and expensive. Moreover, plants contain usually very low contents of these compounds, and therefore other production processes are essential to be developed. Biotechnological production using plant cells as real green factories is a very promising technology, but currently there are still many limiting factors, mainly related to our poor understanding how the plants synthesize these high-value compounds and how the synthesis is regulated.

In the following sections, an overview is given how secondary metabolites are produced in plant and tissue cultures, how the production can be enhanced by classical optimization methods, and what metabolic engineering has to offer today and in the future. Spectacular advances in plant genomics and metabolite profiling offer unprecedented possibilities to explore the extraordinary complexity of the plant biochemical capacity. State-of-the-art genomics tools can be used to engineer the enhanced production of known target metabolites or to synthesize entire novel compounds by the so-called combinatorial biochemistry in cultivated plant cells. Finally, some future perspectives are given for novel techniques and tools that are just now emerging.

## High-Value Products from Medicinal Plants

### Medicinal Plants

Many plants such as crops play a central role in our everyday diet. The nutritional value of edible plants and their constituents has been studied for decades. Besides the edible plants, there is a huge variety of toxic plants in the plant kingdom. These include, for example, many alkaloid or terpene containing medicinal plants such as *Atropa belladonna*, *Camptotheca acuminata*, *Capsicum annuum*, *Catharanthus roseus*, *Erythroxylum coca*, *Papaver somniferum*, *Cannabis sativa*, *Artemisia annua*, and *Taxus* species – just to name a couple of them. These plants have been and still are an important source of pharmaceuticals. Molecules derived from medicinal plants make up a sizable proportion of known drugs currently available on the market. These include compounds such as morphine, codeine, and several anticancer drugs such as paclitaxel, vincristine, and vinblastine, the monetary value of which is very high. In Western medicine, over 25% of prescription drugs sold in pharmacies contain at least one active principle which is directly or indirectly (via semi-synthesis) a natural product. This number does not include the over-the-counter sold drugs or pure phytopharmaceuticals.

According to WHO, 11% of the current 252 drugs considered essential for humans are exclusively derived from flowering plants. Furthermore, plants are also important source of new drug lead compounds. During the past 25 years, 1,010 new drug entities (NDEs) were introduced to the market; 27% of them were either natural products or derived from natural products as semi-synthetic derivatives [3]. In addition, 15% of the drugs were synthesized after the molecule was first discovered from natural resources. Table 1 shows the origin of the 458 NDEs representing the four major therapy groups with anti-infectives (antibacterial, antiviral, antifungal, and antiparasitic), anticancer, antihypertensive, or anti-inflammatory activities discovered between 1981 and 2006. It is remarkable that over 68% of all antibacterial compounds and 51% of all anticancer drugs were directly or indirectly derived from natural resources. Natural sources will undoubtedly continue to play a prominent role in the discovery of pharmaceuticals in the future.

### Secondary Metabolism in Plants

Secondary metabolites are low molecular weight compounds found in small quantities throughout the whole plant kingdom. They exhibit many biological functions vital for the survival of the plant such as responding to stress, mediating pollination, or acting as defense compounds. In plant cell, they are accumulated often in the vacuoles. Besides the importance for the plant itself, secondary metabolites have always been of interest to humans as flavors, fragrances, dyes, pesticides, and pharmaceuticals. However, for most of the secondary metabolites, the exact function in plants still remains unknown.

**Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Table 1** Number of new drug entities (NDEs) discovered during 1981–2006 belonging to the four most important therapy groups (modified from [3])

| Therapy group | Total | N | ND | NS | B | S | V | N+D+NS | % |
|---|---|---|---|---|---|---|---|---|---|
| Antimicrobial | 230 | 12 | 74 | 34 | 13 | 60 | 37 | 120 | 52.2 |
| Anti-bacterial | 109 | 10 | 64 | 1 | 0 | 23 | 11 | 75 | 68.8 |
| Anti-fungal | 29 | 0 | 3 | 0 | 1 | 25 | 0 | 3 | 10.3 |
| Anti-viral | 78 | 0 | 2 | 31 | 12 | 8 | 25 | 33 | 42.3 |
| Anti-parasitic | 14 | 2 | 5 | 2 | 0 | 4 | 1 | 9 | 64.3 |
| Anti-cancer | 100 | 9 | 25 | 17 | 17 | 30 | 2 | 51 | 51.0 |
| Anti-hypertensive | 77 | 0 | 2 | 34 | 0 | 41 | 0 | 36 | 46.8 |
| Anti-inflammatory | 51 | 0 | 13 | 0 | 1 | 37 | 0 | 13 | 25.5 |
| Total | 458 | 21 | 114 | 85 | 31 | 168 | 39 | 220 | 48.0 |

*N* natural product, *ND* natural product derivative, *NS* product is synthesized but the original molecule is discovered from natural sources, *B* biotechnologically produced compound (often a large molecule, protein), *S* synthetic molecule, *V* vaccine

More than 200,000 secondary metabolites have hitherto been discovered from the plant kingdom, but only half of them are structurally fully elucidated [4–6]. They are characterized by an enormous chemical diversity, and every plant has its own characteristic set of secondary metabolites. The production of specific alkaloids is often strongly restricted to certain plant families, whereas, for example, flavonoids are abundant in many plant species. Based on their biosynthetic origins, plant secondary metabolites can be structurally divided into five major groups: polyketides, isoprenoids (e.g., terpenoids), alkaloids, phenylpropanoids, and flavonoids [7]. The polyketides are produced via the acetate-mevalonate pathway; the isoprenoids (terpenoids and steroids) are derived from the five-carbon precursor, isopentenyl diphosphate (IPP) produced via the classical mevalonate pathway, or the novel MEP pathway (see the details in section "Targeting the Metabolic Enzymes"); the alkaloids are synthesized from various amino acids; phenylpropanoids are derived from aromatic amino acids phenylalanine or tyrosine; and the flavonoids are synthesized by the combination of phenylpropanoids and polyketides [8].

Since the discovery of the opium alkaloid morphine almost two centuries ago, alkaloids are still one of the most studied groups of plant secondary metabolites although terpenoids are the largest chemical family of secondary metabolites. It is somehow surprising that such an extensive array of different nitrogen-containing organic molecules are known in higher plants even though only 2% of the plant dry weight is composed of the element nitrogen. The largest requirement of nitrogen is the synthesis of amino acids which function as building blocks of proteins as well as precursors to many secondary metabolites. Alkaloids are thus the most prominent nitrogenous compounds with diverse, complex structures and often possessing strong physiological properties leading their wide use as pharmaceuticals. Human use of them dates back to more than 3,000 years. Currently, more than 12,000 alkaloids are known and they are classified into several subclasses based on the amino acids from which they are derived and according to their chemical structures [9].

At the present time, small amounts of plant compounds including alkaloids, for example, morphine, scopolamine, and vincristine are isolated with often some difficulties from natural vegetation or cultivated plants which explain the high price of the raw material. Numerous secondary metabolites have also served as models for modern synthetic pharmaceuticals [3]. However, the biosynthetic pathways leading to their formation in plants are often long, complex multistep events catalyzed by various enzymes, and are still largely unknown in enzymatic and genetic level.

The best characterized pathways after the decades' intensive classical biochemical research are the biosynthesis of opium and terpenoid indole alkaloids.

Besides the low quantities of the compounds in plants, the production rates may vary from year to year and secondary metabolites often accumulate in specific plant organs in particular time of the vegetative stage of the plant. Some substances can only be isolated from extremely rare plants which is not a choice for sustainable production. Therefore, alternative production systems for plant-derived compounds are needed. The biotechnological production, that is, producing the plant secondary metabolites in cultured plant cells in large bioreactors may offer an attractive alternative approach.

## Biotechnological Production Options

The production of a secondary metabolite of interest for industrial needs is often a challenge. As explained above, these compounds accumulate in plants in small quantities. The biotechnological production of high-value plant secondary metabolites therefore is a viable option to isolation processes from the intact plants or to the total chemical synthesis.

Biotechnology focuses on the exploitation of metabolic properties of living organisms for the production of valuable products of a very different structural and organizational level for the benefit of humans. The organisms vary from microbes (bacteria, fungi, yeast) to plants and animals. Over the decades, many laboratories all over the world have studied the possibilities to produce desired secondary metabolites using plant cell or tissue cultures. Cell cultures have been established from many plants, but often they do not produce sufficient amounts of the required secondary metabolites or the production is unstable. Various classical optimization tools have been applied (see in detail section "Enhancing the Production by Classical Optimization"), but very few success stories exist contrary to many good examples using microbial production systems.

Molecular biology of plants has emerged enormously during the past decades, but still the plant metabolic engineering has met only limited success, again in sharp contrast to microorganisms. This is due to our limited knowledge on complex biosynthesis of secondary metabolites. Despite the rapid development of not only plant genomics but also of analytical tools, genetic maps of biosynthetic pathways are far from complete. Furthermore, regulation of the individual steps leading to the desired end product is poorly understood (section "Metabolic Engineering").

**Plant Cell Cultures** Plant cell culture is a method where plant cells are cultivated under sterile conditions *in vitro*. Commonly, cell cultures are established from callus tissues by cultivating callus in liquid medium, and cell aggregates are broken by either mechanically or by orbital shaking in the cultivation vessel. Plant cells are biosynthetically totipotent, which means that each cell in culture retains its complete genetic information and thus is able to produce the same metabolites as the parent plant. Plant cell cultures have been extensively exploited for various biotechnological applications as an alternative to the traditional agricultural cultivation of plants. The use of cell culture systems offers advantages to produce metabolites in a controlled environment, independent of climatic conditions and under conditions in which the different production parameters can be optimized. Plant cell cultures can be categorized in two main classes, differentiated and undifferentiated cell cultures. The former consists of, for example, organs like shoots, roots, or embryos, whereas callus and cell suspension cultures are referred to as undifferentiated cell cultures. Since the first gene transfers in plants in 1983, achieved by four independently working groups [10–13], a number of efficient gene transfer techniques have been developed for genetic engineering of plants. In addition to so-called direct gene transfer techniques (e.g., particle bombardment, electroporation, microinjection), *Agrobacterium*-mediated gene transfer has been the most commonly used method for gene delivery to plants.

**Hairy Root Cultures** *Agrobacterium* (Rhizobiaceae) is a soil bacterium, which is able to deliver its own plasmid-DNA into the nuclear genome of the plant cell. The bacterium attaches into the wound site of the plant tissue and recognizes certain wound substances, for example, acetosyringone, secreted by the plant [14]. As a result, the *vir* (virulence) region of the plasmid becomes activated and processing of the T-DNA (transferred DNA) for the gene transfer starts [14, 15].

After successful integration of the bacterial DNA into the host plant genome, the tumor formation in the wound site begins as well as the production of low molecular weight tumor substances called opines. The opines are used as a nutrient for the bacterium [16]. The host range of *Agrobacterium* is perhaps broader than that of any other plant pathogenic bacterium, although a number of cultivated monocotyledonous plants and legumes are not natural hosts for this bacterium. The molecular mechanism of the resistance to *Agrobacterium* is not known, although the production of antimicrobial metabolites [17], a lack of *vir* gene inducers [18], inefficient T-DNA integration [19], and *Agrobacterium*-induced programmed cell death [20] have all been suggested. Successful gene transfer in monocot plants via *Agrobacterium* has been performed with maize, rice, wheat, and barley [21].

Hairy root is a plant disease caused by the infection of *Agrobacterium rhizogenes* carrying Ri (root-inducing) plasmid. During infection of the plant, the T-DNA of the Ri-plasmid is transferred and integrated in the nuclear genome of the host. As a result of the transformation, hairy roots appear at the infection site [22]. In the T-DNA, there are four genetic loci, called *rol*A, *rol*B, *rol*C, and *rol*D, which are responsible for the hairy root phenotype. These genes were shown to positively affect the secondary metabolite production in *Nicotiana* [23] and in *Atropa* [24]. Hairy roots are able to grow without externally supplied auxins, and certain *aux* genes from *Agrobacterium* have been shown to provide transformed cells with an additional source of auxin [25]. This is a clear advantage when considering the costs for large-scale cultivation. Hairy roots characteristically lack geotropism and have a high degree of lateral branching. In addition, hairy root cultures have demonstrated their ability to rapidly produce biomass as well as high contents of secondary metabolites, for example, tropane alkaloids [26, 27]. In Table 2, some pharmaceutical compounds produced by hairy root cultures are presented. Unlike crown gall tumors, hairy roots are capable of spontaneously regenerating into plants [57].

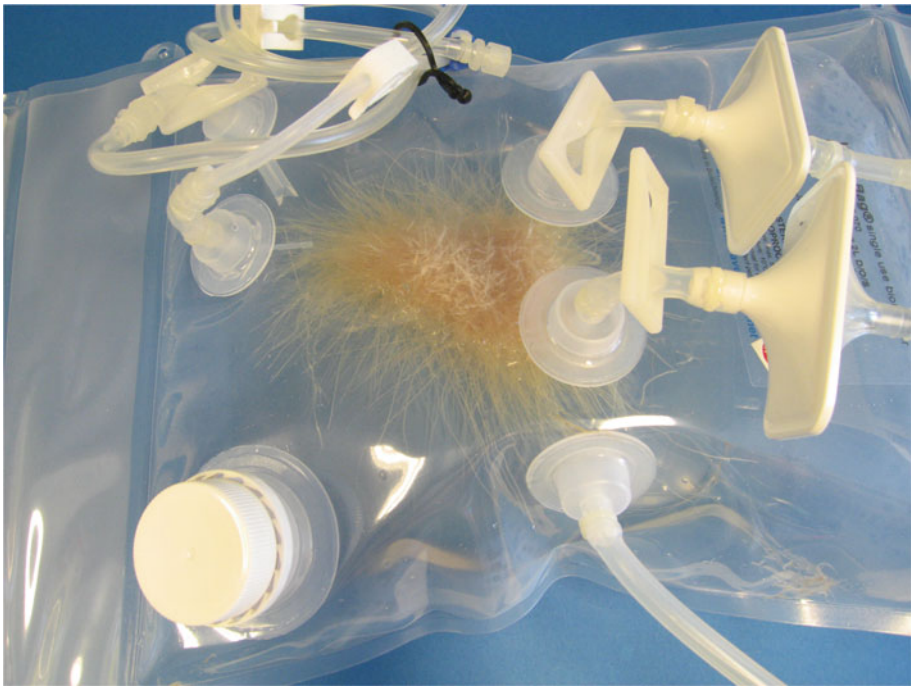
**Bioreactors** The selection of a suitable bioreactor type for the specific process depends on the desired product and the production material, for example, whether the production involves growing undifferentiated cells, hairy roots, or plantlets. Plants cells are larger in size than those of microbial cells, making them more sensitive to shear forces. For this reason, bioreactors have been designed where conventional mechanical impeller stirring have been replaced by bubble or wave-type agitation. Most widely used bioreactors are stirred tanks [58], but also airlift and bubble column reactors have been used in cultivation of plant cells. The classical production of shikonin is performed in airlift type of bioreactors. A balloon-type bubble bioreactor has been successfully used for the cultivation of, for example, ginseng roots [59].

One of the more recent developments in bioreactor design for plant cell applications has been the use of disposable bioreactors, usually plastic bags. Major advantages in these are that the capital costs are much lower than that of common stainless steel tanks. The production of glucocerobrosidase used for treating the enzyme deficiency cased in Gaucher's disease is performed in carrot cells grown in disposable bioreactors by Israeli company Protalix Biotherapeutics (www.protalix.com). The only secondary metabolite of pharmaceutical value, paclitaxel (Taxol®), is commercially produced in *Taxus* cells by German company Phyton Biotech (www.phytonbiotech.com). Moreover, lower expenses allow multiple parallel units to be employed, and high sterility requirements are met when there is no need for costly cleaning processes between runs. Disposable bioreactors may consist of a rigid cultivation container (tube, plate, flask, cylindrical vessel) or a flexible container (bag) [60]. Issues restricting the use of disposable bioreactors arise from a limited experience in their usage, insufficient strength of a plastic material, limited applicability of advanced automatization, and lack of suitable disposable sensors. Wave-mixed bioreactors [61], such as BioWave®, are well suited for small- to middle-scale processes for the production (Fig. 1) of, for example, plant-based secondary metabolites and therapeutic proteins, as well as cultivation of hairy roots [62, 63]. One of the highest productivities reported to date for paclitaxel production in *Taxus baccata* cell suspension cultures was achieved with immobilized cells cultivated in BioWave® system [64, 65].

Important factors when designing the cultivation of plant cell suspension cultures in bioreactors include

**Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Table 2** Examples of metabolites produced by transformed hairy root cultures (adopted mainly from [28, 29])

| Metabolite | Species | Activity | Reference |
|---|---|---|---|
| Ajmalicine, ajmaline | *Rauvolfia micrantha* | Antihypertensive | [30] |
| Artemisinin | *A. annua* | Antimalarial | [31] |
| Benzylisoquinoline alkaloids | *P. somniferum; E. californica* | Analgesic, antibiotic | [32] |
| Betalains | *Beta vulgaris* | Antioxidant, colorant | [33] |
| Camptothecin | *Ophiorrhiza pumila; Camptotheca acuminata* | Antitumor | [34, 35] |
| Iridoid glycosides | *Harpagophytum procumbens* | Anti-inflammatory, analgesic, and antidiabetic | [36] |
| 3,4-Dihydroxy-L-phenylalanine | *Stizolobium hassjoo* | Therapeutic agent against Parkinson's disease | [37] |
| Rutin, hispidulin and syringin | *Saussurea involucrata* | Anti-inflammatory, antifungal | [38] |
| Scopolamine, hyoscyamine and atropine | *A. belladonna* | Anticholinergic | [24, 39] |
| Scopolamine and hyoscyamine | *Datura innoxia* | Anticholinergic | [40] |
| Scopolamine and hyoscyamine | *Datura quercifolia* | Anticholinergic | [41] |
| Scopolamine | *Duboisia leichhardtii* | Anticholinergic | [42] |
| Scopolamine and hyoscyamine | *Datura candida* | Anticholinergic | [43] |
| Scopolamine and hyoscyamine | *Datura innoxia* | Anticholinergic | [44] |
| Scopolamine and hyoscyamine | *H. niger* | Anticholinergic | [40] |
| Scopolamine and hyoscyamine | *H. muticus* | Anticholinergic | [26] |
| Scopolamine and hyoscyamine | *H. muticus, Nicotiana tabacum* | Anticholinergic | [45] |
| Scopolamine | *H. niger* | Anticholinergic | [46] |
| Solasodine | *Solanum khasianum* | Steroid hormone precursor | [47] |
| Paclitaxel | *Taxus brevifolia* | Anticancer | [48] |
| Terpenoid indole alkaloids | *C. roseus* | Antitumor | [49] |
| Thiarubrine A | *Ambrosia artemisiifolia* | Antifungal, antibacterail, antiviral | [50] |
| 6-Methoxy-podophyllotoxin | *Linum album; Linum persicum* | Anticancer | [51] |
| Quinine, quinidine | *Cinchona ledgeriana* | Antimalarial | [52] |
| (+) catechin, (−) epicatechin-3-O-gallate, procyanidin $B_2$-3′-O-gallate | *Fagopyrun esculentum* | Antioxidant | [53] |
| Anthraquinone | *Rubia tinctoria* | Antimalarial, antineoplastic | [54] |
| Thiophene | *Tagetes patula* | Anti-inflammatory precursor | [55] |
| Valpotriates | *Valeriana officinalis* | Tranquilizing | [56] |



**Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Figure 1**
Wave bioreactor is used to culture various types of plant cells. This is a 2-L disposable bag in a Wave® reactor containing tobacco hairy roots

guaranteed sterility through the whole process and low-shear mixing allowing still efficient nutrient transport without causing sedimentation or a loss in viability of the cells. In addition, the possibility for application of light induction for heterotrophic, photomixotrophic, and photoautotrophic cultures might be relevant [62]. Major physical process parameters regarding cultivation of plant cell and tissue cultures are temperature, viscosity, gas flow rates, and foaming.

Sometimes the lack of end-product formation may be due to the feedback inhibition, degradation of the product in the culture medium, or due to volatility of the substrates or end products. In such cases, adding of extra phase as a site for product accumulation might lead to increased production of the desired substance [66]. For example, addition of amberlite resin and charcoal resulted in increased accumulation of anthraquinones and vanilla, and coniferyl aldehyde, respectively [67–69]. On the other hand, bioconversion of water-insoluble substrates in cell culture systems can be aided by using cyclodextrins. They form inclusion bodies in their cyclodextrin cavity and by this way increase the water solubility of the substrates [70].

## Enhancing the Production by Classical Optimization

### Selection of High-Producing Lines

Selection of individual plants with desired traits has been a traditional approach in plant breeding. Similarly, high producers have been selected for further use, for example, for cloning and as a starting material for cell cultures. However, cell clones from the same origin may vary considerably in their metabolite production capacities. Selecting high producers is thus a very empirical approach, requiring a huge amount of screening work before good producing individuals are found [71, 72]. In order to obtain good producing cells, mutation strategies or application of various selective agents, such as *p*-fluorophenylalanine [73], 5-methyltryptophan [74], or biotin [75], have been used. Although undifferentiated plant cells can be maintained in an undifferentiated state using

phytohormones, they are not genetically or epigenetically stable. The concept of somaclonal variation was introduced by Larkin and Scowcroft in the beginning of 1980s, standing for the genetic variability in tissue culture–derived plants or cell culture clones [76]. These changes causing the variation can occur as large rearrangements in chromosomal level, for example, changes in chromosome number, karyotype modifications, or changes in gene level.

Somaclonal variation can be exploited when searching for high secondary metabolite producers or high producers of biomass, although a clear disadvantage is that these changes cannot be predicted or controlled and moreover, they are not always stable or heritable. The effect of culture age on growth rates were observed with *Nicotiana plumbaginifolia*, which showed higher growth rates with older cultures compared to newer cultures [77]. These differences were thought to appear as a cause of higher proportion of cells in older cultures exhibiting mutations which elevate cyclin-dependent kinases. Changes in ploidy levels are reported to affect regeneration capacity [78], gene silencing [79], and secondary metabolite production [80, 81]. After choosing good-producing cell lines, cultivation over time requires usually continuous selection in order to maintain high production levels. However, a gradual loss of secondary metabolite productivity over time is an obstacle in the development of commercial plant cell culture production systems [82, 83].

### Optimization of Culture Medium

One of the major advantages in using plant cell cultures is the possibility of controlled and contained production systems. When attempting to reach high production levels, key roles are played by the composition of nutrient medium and other cultivation parameters, such as temperature, light, phytohormones, and gas exchange.

Because the plant cell is a production factory, the first requirement for obtaining high levels of products is the generation of high amounts of biomass or at least enough biomass for economic product yields. Plant cell cultures are usually grown heterotrophically using simple sugars as carbon source, sucrose being the most commonly used. Carbon source effects mainly on primary metabolism and by this way affects the overall productivity with either increased or decreased biomass production. Sucrose level may also have an indirect impact on secondary metabolite production, as inverse correlation between sucrose and hyoscyamine production was observed in *Hyoscyamus muticus* hairy root cultures [84]. This was probably due to the increased glycolysis and respiration rate with simultaneous overriding of secondary metabolite production. Sucrose is commonly applied in approximately 3% (w/v) concentration, but levels as high as 8% (w/v) have shown to increase the accumulation of indole and benzophenanthridine alkaloids in cell cultures of *Catharanthus roseus* and *Eschscholtzia californica*, respectively [85, 86].

Phosphate and nitrogen levels are perhaps the most important macronutrient factors effecting the secondary metabolite formation. Phosphate usually promotes cell growth, but often has been accompanied by lower secondary product formation. In fact, very often cell proliferation has been accompanied by decrease in secondary product formation and vice versa. For this reason, a two-stage cultivation system could be considered, where the cells are first cultivated in the medium optimized for cell multiplication and then transferred into medium limiting the biomass growth whereas enabling maximum product formation. As an example, shikonin produced by *Lithospermum erythrorhizon* in commercial scale by this type of two-phase system [87]. Low phosphate levels often have been correlated with high secondary metabolite formation, for example, in case of alkaloids in *Datura stramonium* [88], *Nicotiana tabacum* [89], and *C. roseus* [90]. Nitrogen is an important building block of amino acids, nucleic acids, proteins, and vitamins. Generally, nitrogen is added in the form of nitrate or ammonium, and the ratio of these salts plays an important role in secondary metabolite production of the plant cells. Reducing the levels of nitrogen generally leads to lower biomass production and thus leads to higher secondary metabolite production, as in the case of anthocyanin production by *Vitis vinifera* [91].

Phytohormones have an extensive effect not only on growth of plant cells, but also on differentiation and secondary metabolite production. Both the type and concentration of auxin and cytokinin as well as their ratio alter the growth and metabolite production dramatically in cultured plant cells. High auxin levels are known to inhibit the formation of secondary

metabolites in a large number of cases, for example, tobacco alkaloids [92] with the simultaneous activation of polyamine conjugate biosynthesis [93]. Sometimes, replacement of synthetic auxin 2,4-D (2,4-dichlorophenoxy acetic acid) by NAA (naphthalene acetic acid) or natural auxin IAA (indole acetic acid) has shown to enhance the production of anthraquinones, shikonin, or anthocyanins [94–96].

Commonly understanding of cell culture behavior has been relied on the measurements of culture average parameters, such as cell density and metabolite profiles. However, due to the nature of plant cell division, in which daughter cells often remain attached through cell wall, aggregates of various sizes in cell suspension culture are formed. Thus, each aggregate is exposed to different microenvironmental conditions with respect to nutrient and oxygen availability between inner and outer regions of the aggregate [97]. Understanding such subpopulation dynamics and cellular variability using tools such as flow cytometry is important in order to control the culture as a whole.

### Effect of Elicitors

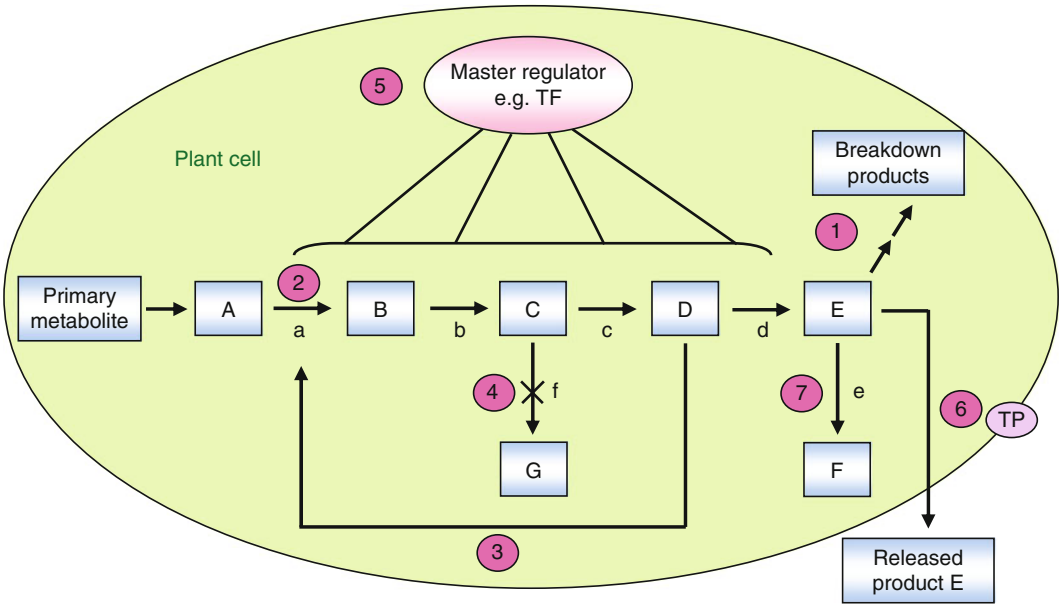
The enhanced production of secondary metabolites from plant cell and tissue cultures through elicitation has opened up a new area of research which could have beneficial influences for pharmaceutical industry. Elicitors are compounds, biotic or abiotic, or even physical factors, which can trigger various defense-related reactions, and thereby induce secondary metabolite formation in plant cells. The mechanisms of how elicitors activate the respective genes and the whole biosynthetic machinery in a plant cell are under active investigation. However, it is evident that the gene expression occurs very quickly after the elicitor contact and many hours before the secondary metabolites are accumulated in a plant cell [98].

In general, elicitors can be categorized based on their molecular structure and origin. Biotic elicitors include compounds such as chitosan, alginate, pectin, chitin or they may contain complex mixtures of compounds like those of fungal or yeast extracts [99]. Abiotic elicitors are chemical compounds of nonbiological origin, for example, heavy metals and vanadate derivatives, or physical factors such as thermal or osmotic stress, UV-irradiation, or wounding. In particular, widely used elicitors for plant cell culture systems are jasmonates and jasmonic acid derivatives, which are naturally occurring hormones involved in the regulation of defence-related genes and act as signaling compounds in these reactions [100]. Application of jasmonates can result in large alterations in desired metabolites in *Catharanthus* [101, 102], in *Taxus* [103], and in *Nicotiana* [98]. Even though plant cells accumulate secondary metabolites typical for species in question independent of the type of elicitor used, the accumulation kinetics may vary greatly with different elicitors. Moreover, elicitors can effect on the release of desired secondary metabolite from the cell to the cultivation medium [104]. This is beneficial when considering the biotechnological production facilitating thus the downstream processing.

Generally, both the elicitor concentration and the length of elicitor application have to be determined for each cell culture individually [104]. Commonly it is thought that the best growth phase for the start of the elicitation is during the exponential growth phase when the enzymatic machinery for elicitor response is most active [105]. In addition, the composition of the culture medium, especially phytohormones, has a major impact on elicitor response. For example, divergent regulation by auxins on the biosynthesis of different metabolites in terpenoid indole alkaloid pathway was observed by *C. roseus* cell cultures [102]. This regulation by auxins was shown to be partly dependent on the presence of methyl jasmonate. Production of various plant-derived medicinal compounds has been successfully induced by using elicitors [106]. Unfortunately, many elicitors also cause a loss of viability of the producing cells, thus a thorough optimization of the whole production process is required when using elicitation.

## Metabolic Engineering

Functional genomics tools offer now huge potential to engineer plant metabolic pathways toward the targeted end product or alternatively to form entirely novel structures through combinatorial biochemistry. However, rational engineering of secondary metabolite pathways requires a thorough knowledge of the whole biosynthetic pathway and detailed understanding of the regulatory mechanisms controlling the flux of the pathway (Fig. 2) [7]. Such information is not




**Medicinal Plants, Engineering of Secondary Metabolites in Cell Cultures. Figure 2**
The hypothetic scheme how the secondary metabolite E could be formed from primary metabolites via different enzymatic steps and how the biosynthesis could be regulated in a plant cell [7]. Engineering of secondary metabolite pathways is a series of complex events. The following strategies could be used to modify the production of hypothetical plant metabolite E: (1) decrease the catabolism of the desired compound, (2) enhance the expression/activity of a rate limiting enzyme, (3) prevent feedback inhibition of a key enzyme, (4) decrease the flux through competitive pathways, (5) enhance expression/activity of all genes involved in the pathway, (6) compartmentalize the desired compound, and (7) convert an existing product into a new product. *TF* transcription factor, *TP* transporter gene

available for vast majority of secondary metabolites, explaining why only limited success has been obtained by metabolic engineering. New genome-wide transcript profiling techniques combined with up-to-date metabolomics allow us now to establish novel gene-to-gene and gene-to-metabolite networks which facilitate the gene discovery also in non-model plants that include most medicinal plants [102]. The ability to switch on entire pathways by ectopic expression of transcription factors suggests new possibilities for engineering secondary metabolite pathways (Fig. 2). Consequently, the utilization of plant cell cultures for biotechnological production of high-value alkaloids would thus become a true viable alternative.

## Gene Discovery

Since the sequencing of *Arabidopsis* genome in 2,000 several other plants are being sequenced but still today very limited information exists for any medicinal plant. Therefore, also the biosynthetic pathways in these plants are largely unknown at the gene level. Several approaches have been developed to identify enzymes and the corresponding genes that are responsible for different biosynthetic pathway steps. One of the classical methods is the identification and isolation of intermediates and enzymes *via* precursor feeding [107]. The other very basic approach is to use cDNA libraries to identify genes by PCR amplification with primers designed to recognize conserved regions on the basis of enzyme homology from other plants with already known sequences [108]. More recently, methods based on differential display comparing mRNA transcripts of elicited and non-elicited cell culture samples have shown their potential in gene discovery. Goossens and coworkers [98] and Rischer and coworkers [102] utilized cDNA-AFLP technique for genome-wide gene hunt, whereas [109] supplemented their search with

homology-based analysis of a cDNA library of elicited cells. In addition, the use of random sequencing of elicited cDNA library can lead to identification of clones involved in the biosynthetic route in question as proven in case of *Taxus* biosynthesis [110].

The use of microarrays as widely used for model plants such as *Arabidopsis* is usually not applicable to medicinal plants simply because none has been sequenced with the very recent exception of tobacco http://www.pngg.org/tgi/index.html. The rapid advance of deep sequencing, however, will soon result in many important species being investigated at genome scale. The 454 pyrosequencing technique is currently perhaps the most widely used so-called next-generation sequencing technique for the *de novo* sequencing and analysis of transcriptomes in non-model organisms like medicinal plants are. For example, the GS FLX Titanium can generate one million reads with an average length of 400 bases at 99.5% accuracy. This technology was successfully used to discover putative genes involved in ginsenoside biosynthesis [111].

Once the candidate genes are discovered, they are functionally tested alone or in combination to find out their real mode of action, for example, improving or altering the production of desired metabolite. This is time consuming, and therefore new high-throughput systems have been developed, for example, miniaturized cell culture formats and multigene transformations.

### Controlling the Expression of Transgenes

In order to be able to modify the metabolite profile of a respective medicinal plant or cell culture, the gene expression of target proteins and enzymes needs to be fine-tuned in an appropriate manner. For that purpose, the elements involved in transcriptional regulation of gene expression should be well characterized and evaluated to ensure correct spatial and temporal display. This also minimizes the potential adverse effects, and the outcome will be as wanted. Specific DNA sequences upstream of the encoding region of a gene that are recognized by proteins (transcription factors) involved in the initiation of transcription are determined as promoters. It is noteworthy that the promoter sequence itself is present in all tissues and cells, and thus the activity is controlled via transcription factors and their abundance. This opens the possibility to boost a cascade of enzymes and influence in the whole biosynthetic pathway in question by overexpressing transcription factors [112].

Promoters used for the metabolic engineering purposes can be divided into three classes:

1. Constitutive, that is, promoters that are continuously on in most or all of the tissues
2. Organ- or stage-specific, that is, promoters controlling spatiotemporal activity of the transgene
3. Inducible that are regulated by an external trigger of chemical or physical nature [113, 114].

As an example of the constitutive promoters and also the most used one in plant genetic engineering is the *Cauliflower mosaic virus 35S* promoter [115, 116]. The CaMV 35S promoter has been very thoroughly characterized and currently a typical CaMV 35S promoter in plant vectors consists of a bit more than one third of the full-length sequence [117]. It has also been observed that a partial duplication from −343 to −90 amplifies expression up to tenfold [118]. This promoter is also the most used one in metabolic engineering of plant cell cultures [119]. For the secondary metabolite production, the hairy root cultures have shown most potent, and little promise has been found with undifferentiated suspension cultures [120]. Actually there exist no studies for trying to find most suitable callus or suspension culture–specific promoters for efficient expression of target genes. This might be one factor why the success in using undifferentiated plant cell cultures for the production of valuable secondary metabolites has been so poor. However, the main blame for this is the current limited understanding of how the metabolic pathways and fluxes of secondary metabolites work in general.

Nowadays that the multigene transformations [121] are paving the way for more accurate and complex engineering of phenotypes, there is also more need to apply different promoter deployment strategies to reach the wanted goals. The delivery of 10–20 genes at the time is already very demanding, and thus there is no space for failure in running their expression. Roughly, two ways of proceeding can be drawn for promoter choice: utilization of the same promoter to run all the genes or combination of promoters to run different target genes in the generated multigene transformants.

The use of same promoter carries the risk of triggering gene silencing. It is very important to increase the promoter diversity via promoter discovery and generation of synthetic sequences to run the expression. Perhaps one of the most interesting ways is to apply bidirectional sequences which allow simultaneous expression of two genes, and thus halves the number of required promoters for multigene engineering [122].

### Targeting the Metabolic Enzymes

From the genetic engineering perspective of medicinal plants, one of the key elements is to express the genes in question in right tissues, and even more importantly target the respective enzymes to correct, specific subcellular compartments. A good example of compartmentalization is the biosynthesis of terpenoids that are synthesized from universal five-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which in turn are formed via two alternate biosynthetic pathways localized in different subcellular compartments. The cytosolic mevalonic acid (MVA) pathway starts with condensation of two molecules of acetyl-CoA into acetoacetyl-CoA and finally gives rise to IPP. The methylerythritol phosphate (MEP) pathway takes place in plastids and leads to the formation of IPP and DMAPP from pyruvate and glyceraldehyde phosphate. The IPP and DMAPP precursors are then processed with prenyl diphosphate synthases in different compartments giving rise to intermediates that serve as substrates to a large group of terpene synthases resulting in construction of the final terpenoids [123, 124]. However, the picture is never black and white, and the subcellular localization studies as well as the genetic engineering experiments have shown that such a thing as a general rule does not apply to all tissues and species. From the rational genetic engineering point of view, this makes things even far more complex and we still need to reveal several aspects of biosynthetic pathways.

Targeting the biosynthetic enzymes to non-original compartment can also lead to interesting results. Precursors can be available in other compartments, and introduction of the respective enzyme can lead to increased accumulation of target compounds. For example, Wu and coworkers [125] showed that redirecting the sesquiterpene pathway from its natural cytosolic location to chloroplasts increased patchoulol accumulation even up to 10,000-fold when compared to native situation. Another example was given by introducing three different targeting modes: cytosolic, plastid, and ER of limonene synthase in transgenic tobacco plants [126]. Both the cytosolic and plastid targeting resulted in limonene formation, whereas ER targeting gave no response probably due to false folding or instability of the protein.

There has also been discussion on so-called metabolic channeling, which means that enzymes from the same pathway, especially the ones committing successive steps, form a protein complex resulting in efficient reactions and regulation of the pathway [127–129]. Aharoni and coworkers [130] interpreted that this might be a cause why some pathways do not seem to proceed even though substantial amount of substrate seem to be available. As a solution, an artificial channeling is suggested with the help of fusion constructs to be applied in the metabolic engineering. These studies also highlight the need for fluxomics and thorough understanding of metabolic pathways (see Sect. "Controlling the Expression of Transgenes").

### Multigene Transformation

The first multigene-carrying transgenic plants were created either with several rounds of crossings between transgenic lines or by transforming transgenic plants with a new set of genes [131, 132]. The current multigene delivery systems are co-transformations with either linked or unlinked genes, that is, genes within a same vector or different vectors, respectively. The transfer itself is carried out either via *Agrobacterium*-mediated or direct transformation techniques. These systems have been developed mainly with crop plants, and the target pathways have been on nutritional composition like in engineering of the carotenoid pathway [133, 134]. These pioneer works have opened the possibility to engineer metabolic pathways of medicinal plants, and the potential in these can be seen almost as limitless. The future aim is the creation of a SMART locus (stable multiple arrays of transgenes), that is, a transgenic locus containing multiple genes, thus avoiding segregation in meiosis and possibly also minimizing rearrangements and silencing [121]. For medicinal plants, the possibility to modify entire

metabolic pathways, to introduce completely new pathways, and to study complex metabolic control circuits and regulations are perhaps the main future goals.

### New Compounds by Engineered Enzymes/Proteins

In most common approaches, the intention of metabolic engineering is to either overexpress or repress genes leading to the accumulation of certain compounds (Fig. 2). The first successful genetic engineering approach to the medicinal plant was performed already almost 20 years ago. Yun and coworkers [135] introduced the gene-encoding hyoscyamine-6β-hydroxylase (H6H) from *Hyoscyamus niger* to the medicinal plant *A. belladonna*. As a result of the overexpression of *h6h*, the plants produced almost exclusively scopolamine, whereas in the control plants the production of hyoscyamine (precursor of scopolamine) was dominant. Later, the function of the same gene was demonstrated to be different in hairy roots of *Hyoscyamus muticus* [26]. The overexpression of *h6h* caused 100-fold increase in scopolamine production, whereas the hyoscyamine contents were not reduced.

There are also examples where genetic engineering can lead to formation of entirely new metabolites. Classically, this can, for example, be achieved by generating somatic hybrids, that is, by exposing enzymes and regulators derived from different genomes to new environments. A good example is the production of demissidine in somatic *Solanum* hybrids neither parent of which contained this specific metabolite but only a set of different precursors [136].

More recently, the combinatorial biochemistry concept which is based on the fact that enzymes often show relaxed substrate specificity, that is, that they can under certain conditions process substrates which differ from the preferred one is exploited in a stricter sense. Usually, native genes are modified with the aim of creating modified enzymes catalyzing new reactions. Initially, attempts to alter the substrate specificity of plant-derived terpenoid synthases by rather unspecific methods such as mutagenesis or truncation were quite unpredictable [137]. Meanwhile, however, it could be shown that preselection of a mutant strictosidine synthase with a specific point mutation according to substrate acceptance results in quite predictable events. *C. roseus* hairy roots expressing the gene formed unnatural terpenoid indole alkaloids when were fed with derivatized precursors in contrast to the wild type [138].

## Future Directions

Different omics in techniques have opened totally new avenues to discover genes, to learn about their functions, for example, transcription, and to finally map the biosynthetic pathways leading to the formation of important secondary metabolites. Metabolomics, which deals with all cellular metabolites, was first defined in microbiology but has also been recognized as an important sector of post-genome plant science [139]. Even in the absence of any visible change in a cell or individual plant, metabolomics, which allows phenotyping by exhaustive metabolic profiling, can show how cells respond as a system. Plant metabolomics is of particular importance because of the huge chemical diversity in plants compared to microorganisms and animals [140]. The number of metabolites from the plant kingdom has been estimated at 200,000 or even more [6], and each plant has its own complex set of metabolites. By integrating transcriptome and metabolome data, one can build networks and get insight on how particular metabolites are formed in plants [102, 140]. This in turn helps us to identify the key genes that could be engineered for the production of improved medicinal plants.

Since cell physiology involves dynamic rather than static processes, the investigation of fluxes is needed to complement phenotyping by metabolomics which only allows inventory, although time-resolved snapshots. However, in contrast to mammalian and microbial cells, flux quantification in plants is much less advanced. This is mainly due to the high degree of subcellular compartmentation and the complexity which arises from intercompartmental transport. Labeling experiments have been very successfully used already in the past for the elucidation of biosynthetic pathways in plants [141], but flux determination has only recently gained pace due to the fast development of analytical and computational technology. Analytical techniques of choice are nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS) [142]. Generally, there are two fundamentally different methods

available facilitating flux measurement – steady-state and dynamic analysis – both of which have certain restrictions and benefits [143]. The latter, that is, kinetic approach is particularly interesting in the sense that it potentially could lead to predictive modeling in regard to secondary metabolism, while steady-state analysis is mainly used to measure carbon flux in well-defined pathways of primary metabolism [144].

In conclusion, modern genomic tools allow for mass gene discovery from plants although many biosynthetic pathways are incompletely resolved and medicinal plants have rarely been sequenced. Nevertheless, predictive metabolic engineering remains a goal of the future. This is because transgene integration in higher plants occurs through illegitimate rather than homologous recombination. DNA integration is random with a preference for gene-rich regions. Gene disruptions, sequence changes, and the production of new proteins constitute common consequences resulting in either predictable or unpredictable effects [145]. In this situation, the power of functional genomics tools allowing the comprehensive investigation of biological systems cannot be overemphasized. Genomics identifies all genes of a plant, while transcriptomics and proteomics provide information about their activities in cells or organs under certain conditions, and finally metabolomics and fluxomics account for the accumulation and kinetics of metabolites, that is, the phenotype. The individual techniques as such are thus invaluable to assign functions, but the real advantage lays in their combination, that is, the systems biology approach [140]. Interestingly at the same time, these tools allow not only the investigation of artificial situations generated by man but also for the first time broad assessment of natural variation.

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# Molecular Breeding Platforms in World Agriculture

Jean-Marcel Ribaut, Xavier Delannay, Graham McLaren, Frederick Okono
Integrated Breeding Platform, Consultative Group on International Agricultural Research, Generation Challenge Programme, c/o CIMMYT, Texcoco, Edo. Mexico

## Article Outline



## Glossary

**Analytical pipeline** A sequence of data management and statistical analysis algorithms which can be applied to one or more data sets to produce a result which can be interpreted and applied in decision making.

**Capacity building** Assistance that is provided to entities, usually institutions in developing countries, which have a need to develop a certain skill or competence, or for general upgrading of capability.

**Cyberinfrastructure (CI)** Computer-based research environments that support advanced data acquisition, data storage, data management, data integration, data mining, data visualization, and other computing and information processing services over the Internet. In scientific usage, CI is a technological solution to the problem of efficiently connecting data, computers, and people with the goal of enabling derivation of novel scientific theories and knowledge.

**Gene** Segment of DNA specifying a unit of genetic information; an ordered sequence of nucleotide base pairs that produce a certain product that has a specific function.

**Information system (IS)** An integrated set of computing components and human activities for collecting, storing, processing, and communicating information.

**Integrated breeding platform (IBP)** Term to describe a Molecular Breeding Platform (see below) in a broader sense including the availability of tools and services suitable for conventional breeding based on phenotypic selection only.

**Molecular breeding (MB)** Identification, evaluation, and stacking of useful alleles for agronomic traits of importance using molecular markers (MMs) in breeding programs. MB encompasses several modern breeding strategies, such as marker-assisted selection (MAS), marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), and genome-wide selection (GWS).

**Molecular breeding platform (MBP)** A term that has come to indicate a virtual platform driven by modern information and communication technologies through which MB programs can access genomic resources, advanced laboratory services, and analytical and data management tools to accelerate variety development using marker technologies.

**Plant breeding** The science of improving the genetic makeup of plants in order to increase their value. Increased crop yield is the primary aim of most plant breeding programs; benefits of the hybrids and new varieties developed include adaptation to new agricultural areas, greater resistance to disease and insects, greater yield of useful parts, better nutritional content of edible parts, and greater physiological efficiency especially under abiotic stress conditions.

**Quantitative trait locus (QTL)** A region of the genome that contains genes affecting a quantitative trait. Though not necessarily genes themselves, QTLs are stretches of DNA that are closely linked to the genes that underlie the corresponding trait.

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## Definition of the Subject

In the last decade, private seed companies have benefitted immensely from molecular breeding (MB) [1]. A private sector-led "gene revolution" has boosted crop adaptation and productivity in developed countries, by applying and combining the latest advances in molecular biology with cutting-edge information and communication technologies combined with accurate plant phenotyping.

MB allows the stacking of favorable alleles, or genomic regions, for target traits in a desired genetic background thanks to the use of polymorphic molecular markers (MMs) that monitor differences in genomic composition among cultivars, or genotypes, at specific genomic regions, or genes, involved in the expression of those target traits. The use of MMs generally increases the genetic gain per crop cycle compared to selection based on plant phenotyping only, and therefore reduces the number of needed selection cycles, hastening the delivery of improved crop varieties to the farmers.

In contrast to the private sector, MB adoption is still limited in the public sector, and is hardly used at all in developing countries. This is the result of several factors, among which are the following: (1) scientists from the academic world are more interested in discovering new genes or QTLs to be published than in applied biology; (2) until recently access to genomic resources was limited in the public sector, especially for less-studied crops; (3) public access to large-scale genotyping facilities was not easily available; and (4) although a broad set of stand-alone tools are available to conduct the multiple types of analyses necessitated by MB, no single analytical pipeline is available today in the public sector allowing integrated analysis in a user-friendly mode.

The situation is even more critical in developing countries as additional limitations include shortage of well-trained personnel, inadequate laboratory and field infrastructure, lack of ISs with applicable and flexible analysis tools, as well as inappropriate funding – simply put, resource-limited breeding programs. As a result, the developing world has yet to benefit from the MB revolution, and most of the countries indeed lack the fundamental prerequisites for a move to informatics powered breeding.

Under those circumstances, developing and deploying a sustainable web-based Molecular Breeding Platform (MBP) as a one-stop shop for information, analytical tools, and related services to help design and conduct marker-assisted breeding experiments in the most efficient way will alleviate many of the bottlenecks mentioned earlier. Such a platform will enable breeding programs in the public and private sectors in developing countries to accelerate variety development using marker technologies for different breeding purposes: major genes or transgene introgression via marker-assisted backcrossing (MABC), gene pyramiding via marker-assisted selection (MAS), marker-assisted recurrent selection (MARS) and, in a not too distant future, genome-wide selection (GWS).

## Introduction

Since the dawn of agriculture, mankind has sought to improve crops by selecting individual plants with the most desirable characteristics or traits. Agricultural productivity has been progressively enhanced by constant innovation, including improved crop varieties to increase production in specific environments [2]. The major objective of crop improvement is to identify within heterogeneous materials those individuals for which favorable alleles are present at the highest proportion of loci involved in the expression of key traits [3]. The classical plant breeding method is based on increasing the probability of selecting such individuals from populations generated from sexual matings. Selection has traditionally been carried out at the whole-plant level (i.e., phenotype), which represents the net result of genotype and environment (and their interactions). Phenotypic selection has delivered tremendous genetic gains in most cultivated crop species, but is severely limited when faced with traits that are heavily modulated by the environment [4]. In addition, the nature of some traits can make the phenotypic testing procedure itself complex, unreliable, or expensive (or a combination of these).

The recent remarkable development of molecular genetics and associated technologies represents a quantum leap in our understanding of the underlying genetics of important traits for crop improvement. The ongoing revolutions in molecular biology and information technology offer tremendous and

unprecedented opportunities for enhancing the effectiveness and efficiency of MB programs. Indirect selection, based on genetic markers, presents an efficient complementary breeding tool to phenotypic selection. Individual genes or QTLs having an impact upon target traits can be identified and linked with one or more markers, and then the marker loci can be used as a surrogate for the trait, resulting in greatly enhanced breeding efficiency [5–8].

Molecular techniques can have an impact upon every stage of the breeding process from parental selection and cross prediction [9], to introgression of known genes [10] and population enhancement. Selection of beneficial alleles of known genes can be done through marker-assisted selection (MAS) – the selection of specific alleles for traits conditioned by a few loci [10] – or through marker-assisted backcrossing (MABC) – transferring specific alleles of a limited number of loci from one genetic background to another, including transgenes [11, 12]. For marker-assisted population improvement, individuals selected from a segregating population based on their marker genotype are intermated at random to produce the following generation, at which point the same process can be repeated a number of times [13]. A second approach aims at direct recombination between selected individuals as part of a breeding scheme, seeking to generate an ideal genotype or ideotype [14]. The ideotype is predefined on the basis of QTL mapping within the segregating population, combined with the use of multi-trait selection indices that can also consider historical QTL data. This variety development approach is commonly referred to as marker-assisted recurrent selection (MARS) [15–17], or genotype construction. An alternative is to infer a predictive function using all available markers jointly, without significant testing and without identifying a priori a subset of markers associated with the traits of interest. This more recent approach coming from genomic medicine [18, 19], and then applied successfully in animal breeding [20] named genome-wide selection (GWS), also appears to be quite promising in crop improvement [7].

Concomitantly with the evolution of marker technologies becoming increasingly "data rich," the amount of data produced by plant breeding programs has increased dramatically in recent years. Increasingly, the critical factor determining the rate of progress in plant breeding programs is their capacity to manage large amounts of data efficiently and subsequently maximize the timely extraction of meaningful information from that data for use in selection decisions. If genotyping has become less of an issue, the efficient management of genotyping data in a broad sense, including sequence information, is increasingly becoming a major challenge in modern plant breeding. This was recognized early on in the private sector where the establishment of platforms or pipelines integrating field and laboratory processes with powerful data management systems (DMS) that merged and analyzed the data collected at every step and guided the process of crop improvement toward the release of improved cultivars has been the key to successful adoption of MB.

A few initiatives have taken place in the public sector to establish efficient data management or ISs [21, 22]. One of these has been led by several centers of the Consultative Group on International Agricultural Research (CGIAR) which have worked over the past decade, along with advanced research institutes (ARIs) and national agricultural research systems (NARS) in developing countries, to develop an open-source generic IS, the International Crop Information System (ICIS), to handle pedigree information, genetic resource, and crop improvement information [23]. Based on some elements of ICIS, the CGIAR Generation Challenge Programme (GCP, http://www.generationcp.org) has invested in integrating crop information with genomic and genetic information and in using existing or developing new public decision-support tools to access and analyze information resources in an integrated and user-friendly way [24]. Another initiative has been led by Primary Industries and Fisheries (PI&F) of the Queensland Government Department of Employment, Economic Development and Innovation in Australia, which recognized that effective data management is an essential element in obtaining maximum benefit from their investment in plant breeding. In conjunction with the New South Wales Department of Primary Industries (NSW DPI) and more recently Dart Pty Ltd (http://www.diversityarrays.com/) they are in the process of developing a linked IS for plant breeding (Katmandoo) that includes applications for capturing field data using hand-held computers, barcode-based seed management systems, and databases to store and link field

trial data, laboratory data, genealogical data, and marker data [25].

Although an IS involves far more than a database, the development and implementation of a suitable database system alone remains a real challenge because of the fast turnover in technologies, the need to manage and integrate increasingly diverse and complex data types, and the exponential increase in data volume. Previous solutions, such as central databases, journal-based publication, and manually intensive data curation, are now being enhanced with new systems for federated databases, database publication, and more automated management of data flows and quality control. Along with emerging technologies that enhance connectivity and data retrieval, these advances should help create a powerful knowledge environment for genotype–phenotype information [26].

In addition to efficient data management, advances in statistical methodology [27–29], graphical visualization tools, and simulation modeling [9, 30–32] have greatly enhanced these ISs. The availability of molecular data linked to computable pedigrees [33] and phenotypic evaluation now makes genotype–phenotype analysis a practical reality [34].

In order to realize the full potential of marker technologies and bioinformatics in plant breeding, tools for molecular characterization, accurate phenotyping, efficient ISs, and effective data analysis must be integrated with breeding workflows managing pedigree, phenotypic, genotypic, and adaptation data. The goals of this integration of technologies are to (1) create genotype–phenotype trait knowledge for breeding objectives, and (2) use that knowledge in product development and deployment [4].

This entry generally explores the pace of innovation in world agriculture and the rise of MB. It particularly illustrates the accelerating application of information and communication technologies to the information management challenges of MB and, as a result, the emergence of virtual molecular breeding platforms (MBPs) as a vital tool for accelerating genetic gains and rapidly developing more resilient and more productive cultivars.

This entry reviews the rationale for access to MB technology and services and the status of existing public analytical pipelines and ISs for MB, and offers a detailed case study for the CGIAR GCP Integrated Breeding Platform (IBP) – the pioneer public sector MBP specifically targeting developing country breeding programs. It explores the gaps between countries and between crops in the application of informatics-powered MB approaches, and the potential for adopting MBPs to close these gaps; and it reviews institutional, governmental, and public support for these approaches. The entry discusses the challenges and opportunities inherent in MBPs, and the potential economic impact of MB. Finally, the entry explores the future directions and perspectives of MBPs.

## Marker Technologies and Service Laboratories

Markers are "characters" whose pattern of inheritance can be followed at the morphological (e.g., flower color), biochemical (e.g., proteins and/or isozymes), or molecular (DNA) levels. They are so called because they can be used to elicit, albeit indirectly, information concerning the inheritance of "real" traits. The major advantages of molecular over other classes of markers are that their number is potentially unlimited, their dispersion across the genome is complete, their expression is unaffected by the environment and their assessment is independent of the stage of plant development [35]. During the past two decades, DNA technology has been exploited to advance the identification, mapping, and isolation of genes in a wide range of crop species. The first generation of DNA markers, restriction fragment length polymorphisms (RFLPs), was used to construct the earliest genome-wide linkage maps [36] and identify the first QTLs [37, 38]. During the 1990s, emphasis switched to assays based on the polymerase chain reaction (PCR), which are much easier to use and potentially automatable [39]. The development of simple sequence repeats (SSRs) [40], amplified fragment length polymorphisms (AFLPs) [41], and single nucleotide polymorphism (SNP) [42] opened the door for large-scale deployment of marker technology in genomics and progeny screening.

SNPs are amenable to very high throughput and a wide range of detection techniques has been developed for them, from singleplex systems to high-density arrays. They can be used in fully integrated robotic systems going from automated DNA extraction to automated scoring in high-throughput detection platforms. The combination of increase in throughput and lowering in costs makes SNPs highly suitable to

intensive marker applications in plant breeding such as MARS and the emerging approach of GWS. Based on SNP technology, production of molecular marker (MM) data expanded more than 40-fold between 2000 and 2006 at Monsanto, while cost per data point decreased to one sixth of the original cost [43].

With the transition from SSRs to SNPs and the concomitant large increase in the demand for genotyping as markers get more and more widely used in a broad range of applications from medicine to plant breeding, marker genotyping laboratories have evolved from relatively low-tech operations to highly automated, high-throughput laboratories using an array of sophisticated equipment (pipetting robots, high-density PCR, high-throughput SNP detection machines, high-level informatics). Although large private seed companies have had the need and the resources to put in place large-scale genotyping laboratories for their own uses, smaller programs, especially in the public sector, have typically not had the resources or the justification to establish such large operations to respond to their increasing need for SNP genotyping data. In response to this need, a few private marker service laboratories have sprung up over the past few years, which can provide complete genotyping services for their customers, from DNA extraction to generation of large numbers of SNP or other datapoints. Due to their broad customer base (from medical research laboratories to animal and plant breeding operations, both public and private), these laboratories can have a large volume of datapoint production which may lead to low costs for the customer and high throughput. They are able to invest in the most advanced equipment to keep up with the constant evolution of genotyping technologies and are able to pass on the resulting benefits to their customers. Processes have now been put in place for rapid shipment of leaf samples from any location (field or laboratory) around the world without any restrictions. Examples of such companies that can service breeding programs from around the world are DNA LandMarks, Inc. of Saint-Jean-sur-Richelieu, Quebec, Canada (http://www.dnalandmarks.ca/english/) and KBioscience Ltd. of Hoddesdon Herts, UK (http://www.kbioscience.co.uk/). For many public breeding programs and small companies, especially in developing countries, it is now more efficient to use those types of contract genotyping services than to try to support their growing MB needs through the establishment of an in-house laboratory. Functional and reliable SNP laboratories are especially difficult to establish in many developing countries due to the unreliability of the power supply, difficulties in shipping and storing and a low level of resources for the purchase and maintenance of sophisticated equipment. The GCP is facilitating the linkage between users and service laboratories through its marker services, a component of the breeding services offered through the GCP's IBP.

## Analytical Tools, Software, and Pipelines

One of the achievements of the plant biotechnology revolution of the last two decades has been the development of molecular genetics and associated technologies, which have led to the development of an improved understanding of the basis of inheritance of agronomic traits. The genomic segments or QTLs involved in the determination of phenotype can be identified from the analysis of phenotypic data in conjunction with allelic segregation at loci distributed throughout the genome. Because of this, the mode of inheritance, as well as the gene action underlying the QTL, can be deduced [44]. As with the improvement in marker technologies, the statistical tools needed for QTL mapping have evolved from a rudimentary to a very sophisticated level [45]. Previous approaches based on multiple regression methods, using least squares or generalized least squares estimation methods [46, 47], have evolved to composite interval mapping [9], mixed model approaches using maximum likelihood or restricted maximum likelihood (REML) [48], and Markov Chain Monte Carlo (MCMC) algorithms [49, 50], which use Bayesian statistics to estimate posterior probabilities by sampling from the data. In parallel, with progress in the characterization of genetic effects at QTLs and refinement of QTL peak position through meta-analysis [51], advances have also been made in understanding the impact of the environment on plant phenotype. The mapping of QTLs for multiple traits has allowed the quantification of QTL by environment interaction (QEI) [52] and, more recently, approaches using factorial regression mixed models have been applied to model both genotype by environment interaction [53] and QEI [48, 54, 55]. Recent approaches are now

implemented to evaluate gene networking [56] and epistasis, based on Bayesian approaches [57, 58] or through stepwise regression by considering all marker information simultaneously [59, 60]. Epistasis and balanced polymorphism influence complex trait variation [61, 62], and classical generation means analyses, estimates of variance components, and QTL mapping indicated an important role of digenic and/or higher-order epistatic effects for all biomass-related traits in model plants [63] and in crops [64–66]. It will be critical to implement the most efficient MB strategies in order to evaluate and include these genetic effects in breeding schemes [60].

All tools necessary to run MB projects, from the simplest to the most complicated approaches, are available today in the public domain. They are based on different algorithms and statistical approaches, from the very simple to the more complex. One challenge is the diversity of tools available for a given analytical function or along the different steps of an analytical pathway, making the choice of the "right" tool difficult and the move from one analytical step to the next very tedious due to the complete lack of common standards and formatting across tools. The number of applications available for QTL analysis illustrates well the multiplicity and diversity of tools that are available for a given analysis. The following software packages have been developed over the past 20 years:

- Mapmaker/QTL [67]
- MapQTL [68, 69]
- QTL Cartographer [9, 70]
- PLABQTL [71]
- QGene [72, 73]
- Map Manager QT [74]
- iCIM [59, 60]

For most of these applications, the first versions were already available 15 years ago and the multiplicity and possible duplication generated by the independent development of these tools were already identified at the Gordon Research Conference on Quantitative Genetics and Biotechnology held in February 1997 in Ventura, California. A main objective of that workshop was to survey participants on the attributes of several software packages for QTL mapping and to define their analytical needs which were not presently met by the existing software packages. The workshop covered software for QTL mapping in inbred and outcrossed populations and the conclusions are available at: http://www.stat.wisc.edu/~yandell/statgen/software/biosci/qtl.html. In those conclusions one can read that "[a] consensus was reached that there is considerable overlap in the kinds of matings handled and statistics produced by the various QTL mapping software packages," clearly identifying the need for better coordinated efforts. Such coordination never took place, as is often the case in public research. As a result, most of those QTL packages are still available today, although in more sophisticated versions. They are all suitable for QTL mapping but use different statistical algorithms, present a different user interface, and necessitate different input and output file formats.

Some specialists in the field realized that the public software packages are usually too specialized and too technical in statistics to permit a thorough understanding by the many experimental geneticists and molecular biologists who would want to use them. In addition, the fast methodological advances, coupled with a range of stand-alone software, make it difficult for expert as well as non-expert users to decide on the best tools when designing and analyzing their genetic studies. Based on this rationale, a few commercial analytical pipelines emerged about a decade ago that include some of the QTL packages mentioned above. Two of them are Kyazma and GenStat®. These applications assist plant scientists by providing easy access to statistical packages for phenotypic and genotypic data. Kyazma was founded in the spring of 2003 (http://www.kyazma.nl/), and offers powerful methods for genetic linkage mapping and QTL analysis. Since 2003 Kyazma has taken over the development of the software packages JoinMap® and MapQTL® from Biometris of Plant Research International. Kyazma handles the distribution and support of JoinMap and MapQTL and, in collaboration with the statistical geneticists of Biometris, Kyazma provides introductory courses on genetic linkage mapping and QTL analysis in order to make the use of the software even more accessible. GenStat encompasses statistical data analysis software for biological and life science markets worldwide. GenStat includes the ASReml algorithm (average information algorithm for REML) to undertake very efficient meta-analyses of data with linear mixed models. The development of GenStat at Rothamsted

began in 1968, when John Nelder took over from Frank Yates as Head of Statistics. Roger Payne took over leadership of the GenStat activity when John Nelder retired in 1985 (http://www.vsni.co.uk/). An important feature of GenStat is that it has been developed in (and now in collaboration with) a Statistics Department whose members have been responsible for many of the most widely used methods in applied statistics. Examples include analysis of variance, design of experiments, maximum likelihood, generalized linear models, canonical variates analysis, and recent developments in the analysis of mixed models by REML.

These commercial analytical pipelines offer a set of quality tools to researchers in plant science. However, they cover only a part of the configurable workflow system that is required for integrated breeding activities. In addition, there is a need to have tools and analytical pipelines that are freely available and, if possible, based on open source code to avoid dependence on private companies that might discontinue support and ensure access to the tools even with limited financial resources, which is a critical constraint in the arena of research for development, of which breeding programs of developing countries are key partners. It is important to underline that a version of GenStat that does not include the most advanced version of the different tools but allows users to run most basic analyses is available for breeding programs in developing countries. The web site for the GenStat Discovery Edition is http://www.vsni.co.uk/software/genstat-discovery/, but this version of the pipeline does not include QTL selection based on the mixed model approach, which is available in the commercial version.

The issue of open source code is an important one as, even for freely-available tools, the lack of availability of the source code limits the further expansion and customization of the tools. It also reduces the opportunity of researchers in developing countries to participate in methodology development. Over the last decade, a programming language and software environment for statistical computing and graphics, R, is becoming the reference in open source code for a broad range of biological applications, including genetic analysis (http://www.r-project.org/). Its source code is freely available under the *GNU General Public License* (http://en.wikipedia.org/wiki/GNU_General_Public_License). The R language has become a de facto standard among statisticians for the development of statistical software. It compiles and runs on a wide variety of UNIX, Windows, and MacOS platforms. R is similar to other programming languages, such as C, Java, and Perl, in that it helps people perform a wide variety of computing tasks by giving them access to various commands. For statisticians, however, R is particularly useful because it contains a number of built-in modules for organizing data, running calculations on the information, and creating graphical representations of the data sets. R provides a wide variety of statistical (linear and nonlinear modeling, classical statistical tests, time-series analysis, classification, clustering, etc.) [29] and graphical techniques, and is highly extensible. Close to 1,600 different packages reside on just one of the many web sites devoted to R, and the number of packages has grown exponentially. However, R is difficult to use directly and procedures based on R must be wrapped in user-friendly menu systems if field biologists are to use them.

### Information Systems

A functional IS involves far more than an analytical pipeline; it is a complete system that should include:

- A project planning module
- A germplasm management module
- A robust relational database
- Analytical standards
- Data collection and cleaning tools
- Analytical and decision support tools
- Query tools
- A cyber infrastructure (CI) that links the different tools in a cohesive and user-friendly way

Key elements of an IS are obviously the CI and the DMS as described in the following section. The value of an IS does not only reside in the quality of the individual tools or modules that are part of it, but rather in the CI or middleware that ensures cohesion across tools and efficient communication with databases.

There are not many examples of breeding ISs in the public domain. One example is the ICIS (http://www.icis.cgiar.org, [23]). ICIS is an open source IS for managing genetic resource and breeding information for any crop species. It has been developed over the last 10 years through collaboration between centers of the

CGIAR, some NARS, and private companies. The ICIS system is Windows-based, and distributable on CD-ROM or via the Internet. It contains a genealogy management system (GMS, [33]) to capture and process historical genealogies as well as to maintain evolving pedigrees and to provide the basis for unique identification using internationally accepted nomenclature conventions for each crop; a seed inventory management system (IMS); a DMS [75] for genetic, phenotypic, and environmental data generated through evaluation and testing, as well as for providing links to genomic maps; links to geographic ISs that can manipulate all data associated with latitude and longitude (e.g., international, regional, and national testing programs); applications for maintaining, updating, and correcting genealogy records and tracking changes and updates; applications for producing field books and managing sets of breeding material, and for diagnostics such as coefficients of parentage and genetic profiles for planning crosses; tools to add new breeding methods, new data fields, and new traits; and tools for submitting data to crop curators and for distributing data updates via CD-ROM and electronic networks. The community of ICIS collaborators communicates via the ICIS Wiki (http://www.icis.cgiar.org), where all design and development decisions are documented. Feature requests and bug reports are made through the ICIS Communications project and the source code is published through various other ICIS projects on CropForge (http://cropforge.org). A commercial company, Phenome-Networks, has implemented a Web-based IS based on ICIS (http://phnserver.phenome-networks.com/).

Another system available is the Katmandoo Biosciences Data Management System (http://www.katmandoo.org/, [25]), which is a freely available, open source DMS for plant breeders developed by PI&F, NSW DPI, and DArT Pty. Ltd. It comprises linked ISs for plant breeding including applications for capturing field data using hand-held computers, barcode-based seed management systems, and databases to store and link field trial data, laboratory data, genealogical data, and marker data. A particular focus is on the use of whole-genome MM information to create graphical genotypes, track the ancestral origin of chromosomal regions, validate pedigrees, and infer missing data. It includes the applications of the Pedigree-Based Marker-Assisted Selection System (PBMASS) developed by PI&F as well as a seed management system, a digital field book for hand-held computers, and a system for directly recording weights of barcoded samples.

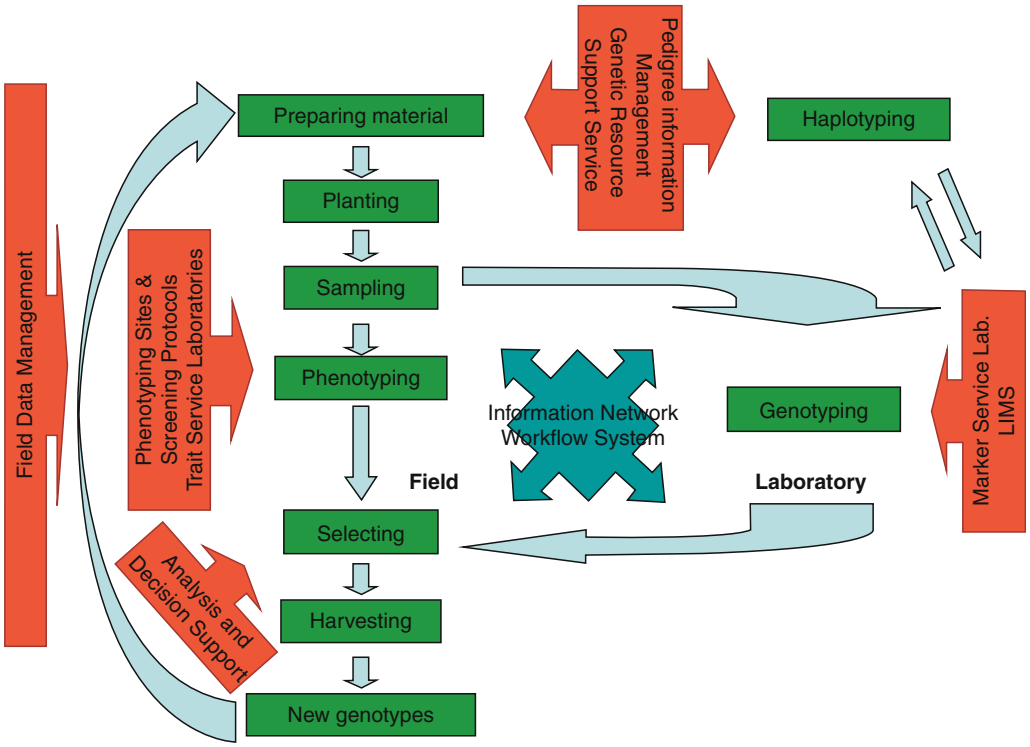
Both ISs struggle with the problem of integrating the different components into a single configurable system which matches the workflows of different breeding projects. Such a workflow should provide the user all tools and analytical means required to run a crop cycle: from germplasm preparation and planting, through the collection of phenotypic and the production of the genotypic data and their analysis, to the identification of genotypes to be crossed or the selection of suitable genotypes to be planted in the next cycle (Fig. 1).

In order to do this effectively, a CI is required which allows syntactic linkage between different data resources and applications.

## Cyberinfrastructure and Data Management

We have referred to the revolution in Information and Communication Technology and the opportunities it presents for improving the efficiency of plant breeding. However, plant breeding is not the only area of biology being affected by this revolution and, in fact, the successful deployment of MB depends on other fields of information-intensive biology delivering knowledge (markers and methodology) to plant breeding. Even more is expected of the information and communications technology (ICT) revolution in the developing world, as it offers an opportunity for scientists there to overcome some of the constraints of isolation, the "brain drain," and the lack of infrastructure which have prevented them from fully participating in science for development in the past [76].

It is generally recognized that upstream biology is increasingly reliant on networks of integrated information and on applications for analyzing and visualizing that information. Discipline-specific (sequence and protein databases) and model organism ISs such as Graingenes (http://wheat.pw.usda.gov/GG2/index.shtml), Gramene (http://www.gramene.org/), MaizeGDB (http://www.maizegdb.org/), and Soybase (http://www.soybase.org/) have been developed to facilitate exchanges in molecular biology and functional genomics. As noted above, plant breeding




**Molecular Breeding Platforms in World Agriculture. Figure 1**
Different activities conducted during the crop cycle of an MB experiment presented in a generic way

depends on these upstream sciences of molecular biology, functional genomics, and comparative biology to deliver the knowledge needed to deploy MB. The bottleneck in the overall network has been the technology needed to integrate diverse and distributed information resources, and many information scientists have been working on this problem [24, 26, 77].

One constraint to integration of scientific information is the necessity to have a standard terminology for biological concepts across species and disciplines. A successful example of such standardization is the Gene Ontology (GO) initiative (http://www.geneontology.org, [78]). Another more specialized ontology initiative, especially pertinent to agriculture, is the Plant Ontology Consortium (POC: http://www.plantontology.org, [79–81]). However, these formal descriptions remain somewhat limited to biology of model plants and controlled environments. A key challenge will be to extend such standards to describe characteristics of plants growing in the unique, stress-prone environments found within the developing world to ensure a wider impact of such standards on international agriculture. The GCP has been working with POC to expand these ontologies to economic traits and farming environments so that they can be used in the field of plant breeding [82].

Another constraint to the efficient utilization of genomic information is the sheer volume of sequence data that can now be generated very cheaply across numerous genotypes. ISs to handle this volume of information are struggling to keep up. In plant biology, some examples of systems aiming to handle these torrents of data are the Germinate database ([83], http://bioinf.scri.ac.uk/public/?page_id=159) and the Genomic Diversity and Phenotype Connection (GDPC, http://www.maizegenetics.net/gdpc/). The primary goal of Germinate is to develop a robust database which may be used for the storage and retrieval of a wide variety of data types for a broad range of plant species. Germinate focuses on genotypic, phenotypic, and passport data, but has been designed to potentially handle a much wider range of data including, but not

limited to, ecogeographic, genetic diversity, pedigree, and trait data, and will permit users to query across these different types of data. The developers have aimed to provide a versatile database structure, which can be simple, requires little maintenance, may be run on a desktop computer, and yet has the potential to be scaled to a large, well-curated database running on a server. The design of Germinate provides a generic database framework from which interfaces ranging from simple to complex may be used as a gateway to the data. The data tables are structured in a way that they are able to hold information ranging from simple data associated with a single accession or plant, to complex data sets, images, and detailed text information. Features of the Germinate database structure include its ability to access any information associated with a group of accessions and to relate different types of information through their association with an accession. The GDPC database was designed as a research database to support association genetics applications such as Tassel (http://www.maizegenetics.net/index.php?option=com_content&task=view&id=89&Itemid=119) and is being extended to handle higher and higher densities of genotyping and sequence data. The second version of Germinate seems quite similar to GDPC and if new databases are developed to handle the large data files to be generated soon through high-throughput sequencing, some conversion tools should be easily developed to migrate data from one system to another.

Finally, the problem of integrating all these diverse and widely-distributed information resources is a major informatics challenge, which is being tackled on several fronts at several levels of complexity. The BioMOBY project ([84], http://www.biomoby.org, [85]) and the Semantic Web seek to define standards that will allow computer programs to interpret requests for information or services, find informatics resources capable of fulfilling those requests, and return the results without the authors of the interacting software having specifically collaborated. In the private sector, solutions have been more pragmatic and Enterprise Software solutions have been developed to link data resources and applications with specific services. The iPlant Collaborative (http://www.iplantcollaborative.org/) is a National Science Foundation (NSF)-funded initiative designed to bring these Enterprise Software solutions to the biological sciences in the form of CI which can support any biological data resource and analytical application. iPlant and the GCP are collaborating on integrating plant breeding information resources and applications into the infrastructure. This will automatically link these resources to upstream biological applications using the same infrastructure such as that used by the Systems Biology Knowledgebase initiative (http://genomicscience.energy.gov/compbio/#page=news) of the US Department of Energy which will be producing knowledge needed for crop improvement.

With all the progress achieved in marker technology, software development, analytical pipelines, and DMS, it is time to provide an IS, available through a public platform, that will offer breeding programs in developed and developing countries access to modern breeding technologies, in an integrated and configurable way, to boost crop quality and productivity.

## Case Study: GCP's Integrated Breeding Platform

To fill this gap in the public sector and in particular in the arena of research for development, the GCP has been coordinating the development of the IBP (www.generationcp.org/ibp) in collaboration with scientists from ARIs, CGIAR centers, and national research programs since mid-2009. In a first phase the IBP aims at serving the needs of a set of 14 pioneer "user cases" – MB projects for eight crops in 16 developing countries in Africa and Asia. Leading scientists of those user cases help in testing the prototypes developed for the different tools of the analytical pipeline and contribute to the monitoring and evaluation of the platform development. This ensures that IBP development is driven by real breeding needs and its interface is user-friendly.

### Objective of the IBP

The overall objective of the IBP project is to provide access to modern breeding technologies, breeding material, and related information and services in a centralized and functional manner to improve plant breeding efficiency in developing countries and hence

facilitate the adoption of MB approaches. The short-term objective of the project (the initial phase) is to establish – through a client-centered approach – a minimum set of tools, data management infrastructure, and services to meet the needs and enhance the efficiency of the 14 user cases.

To achieve the overall objective, GCP is developing and deploying a sustainable IBP as a one-stop shop for information, analytical tools, and related services to design, implement, and analyze MB experiments. This platform should enable breeding programs in the public and private sectors to accelerate variety development for developing countries using marker technologies – from simple gene or transgene introgression to gene pyramiding and complex MARS and GWS projects. Hence IBP aims at bringing cutting-edge breeding technologies to breeding programs that are too resource-restricted to invest in the requisite genotyping and data management infrastructure and capacity on their own.

## The IBP Partnerships

The primary stakeholders of the platform are plant scientists – at this time specifically breeders leading the selected MB projects of the 14 pioneer user cases. These pioneer user cases are all recently initiated marker-assisted breeding projects with specific budgets, objectives, and work plans. The needs of the projects are defining the user requirements, and hence the design and development prioritization of the different elements of the platform. In selecting the user cases, crop diversity was a primary consideration, since the platform is supposed to address the needs of a broad variety of crops. The platform's reciprocal contribution to these breeding projects is in helping them overcome bottlenecks that would compromise final product delivery and in enhancing their overall efficiency and chances of success by providing appropriate tools and support.

The developmental phase of the IBP brings together highly regarded public research teams – institutes and individuals who have been working on the challenges of crop information management and analysis, biometrics, and quantitative genetics. This team of bioinformaticians, statisticians, and developers aims to design and develop the different elements of the platform, based on needs and priorities defined by the user cases.

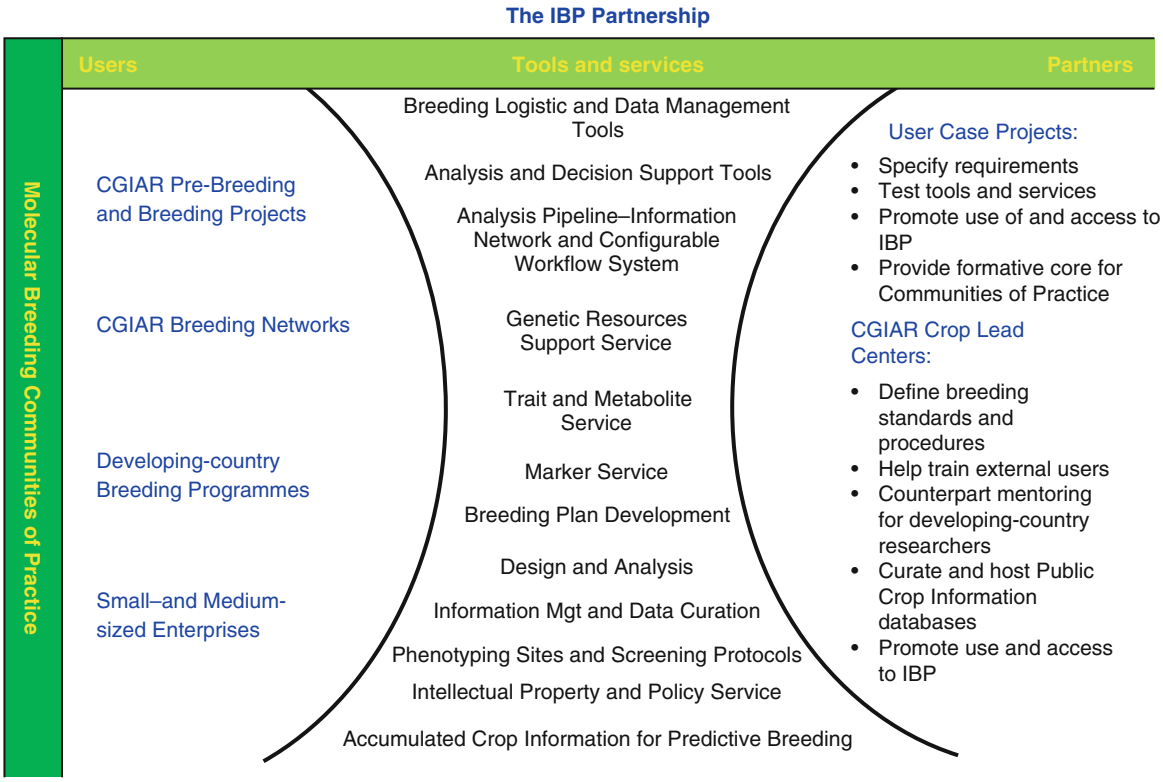
A continuous dialogue between users, developers, and service providers ensures a healthy balance between having a user-driven platform on the one hand, with a reasonable degree of "technology push" on the other hand, to ensure that users are kept abreast of technological solutions they may not be aware of but that would facilitate and accelerate breeding work.

The private sector has led the application of MB approaches and utilization of MBPs. The IBP is the first public sector effort of this magnitude aimed at developing and deploying an MBP. Given that MB for complex polygenic traits, and more so MARS, is still in its infancy in the public sector, it is recognized that efficient partnerships with the major private sector transnational seed companies is a strong prerequisite for the success of the IBP project. Consultations are ongoing with leaders in MB at Limagrain, Monsanto, Pioneer-DuPont, and Syngenta. Partnership with the private sector includes mainly some technology transfer, especially for stand-alone tools, and access to human resources to advise on the development of the platform and contribute to developing new tools or implement data management. The users, tools and services, and partnership of the platform are presented in Fig. 2.

## The Platform

The IBP has three broad components (see Fig. 3): a Web-based portal and helpdesk, an open-source IS incorporating an adaptable breeding workflow system, and breeding and support services.

The stepwise development of the breeding workflow includes: (1) access to existing tools, (2) development of stand-alone new tools or adapted versions of existing tools to address the needs of the user cases, and (3) the integration of those tools into a CI (collaboration with the iPlant initiative) or through a thin middleware linking with local database to form a user-friendly configurable workflow system (CWS). A first version of the CWS, including an adequate set of tools, should be available by mid-2012, with full unfettered access scheduled for 2014.




**Molecular Breeding Platforms in World Agriculture. Figure 2**
The IBP partnership

### Component 1: The Integrated Breeding Portal and Helpdesk

Inaugurated by mid-2011, the portal is the online gateway through which users access all the tools and services of the IBP. Through the portal, users will select and download tools and instructions, order materials, and procure laboratory services.

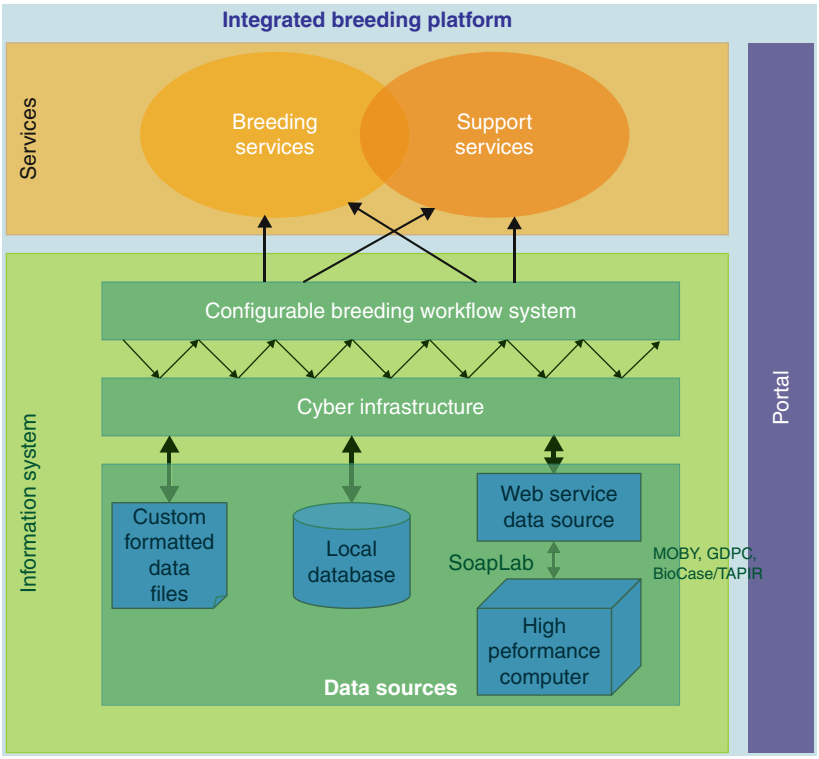
The portal's helpdesk facilitates its use and ensures access for users who cannot efficiently use the Web interface by providing the elements they need via email, compact disc, and other offline media.

Through their user-friendly networking components, the Portal and Helpdesk will stimulate the development of collaborative crop-based and discipline-based communities of practice (CoPs). The CoPs are expected to promote the application of MB techniques and the utilization of facilitative information management technologies, enhance data and germplasm sharing, and generally advance modern breeding capacity by linking CGIAR Centers and ARIs with developing-country breeding programs and research organizations. There is a strong hope that CoPs will facilitate and accelerate a paradigm shift to a more collaborative, outward-looking, technology-enhanced approach to breeding.

### Component 2: The Information System

The IBP IS is structured as a CWS, with access to both local databases and distributed resources, such as central crop databases, molecular databases from GCP partner sites and from public initiatives such as Gramene and GrainGenes.

**The Configurable Workflow System** This CWS is the operational representation of the IS and will be implemented by assembling informatics tools into applications configured to match specific breeding workflows (e.g., for MAS, MABC, or MARS; Fig. 4). The tools are organized in a series of functional modules comprising




**Molecular Breeding Platforms in World Agriculture. Figure 3**
The IBP and its three main components

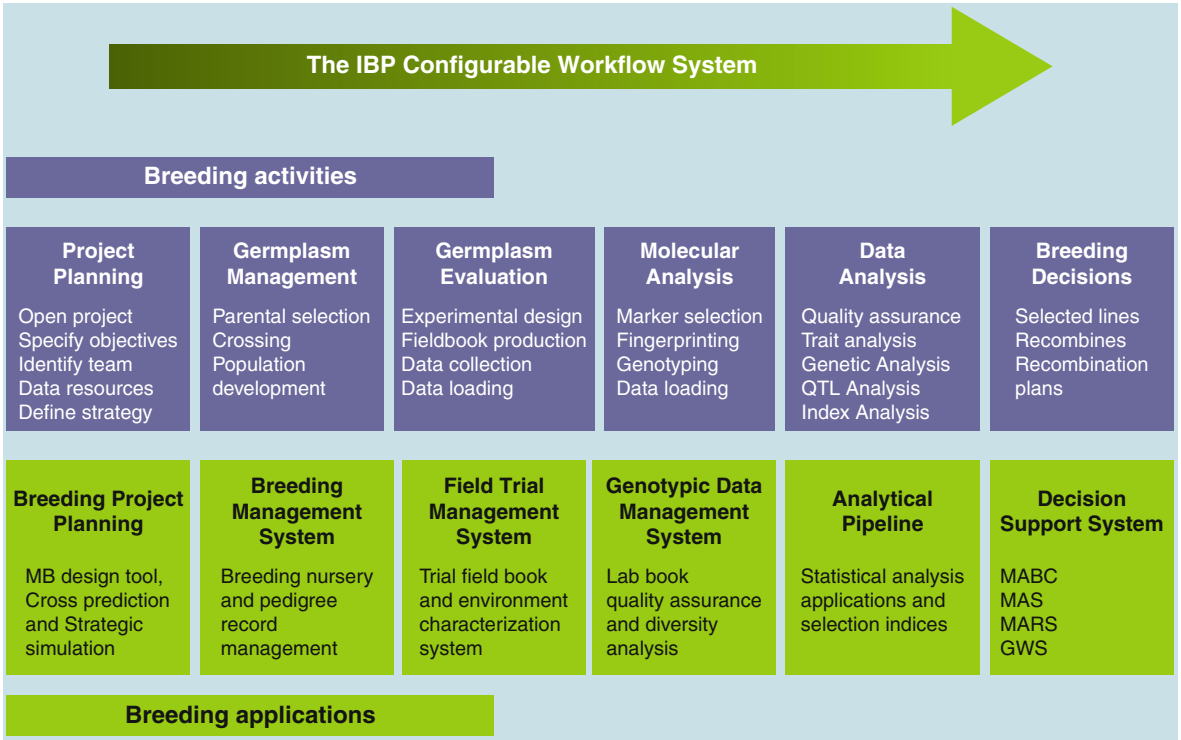
the Integrated Breeding Workbench, which is really the background structure that implements the CWS.

The IBP CWS drives the users through the different practical steps or activities of an MB project. The setup of the experiment and the germplasm management are the first steps of any project, to be followed by a set of activities that can be repeated during subsequent crop cycles, depending on the breeding objective of the experiment:

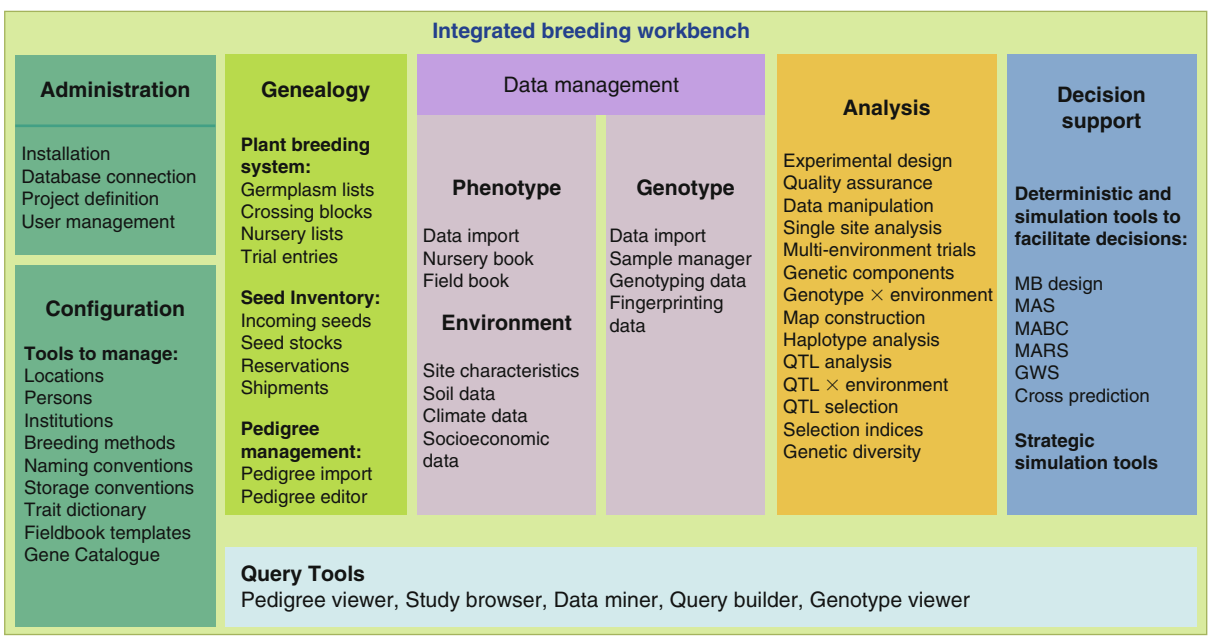
- Germplasm evaluation
- Genetic analysis
- Data management
- Data analysis, and
- Breeding decisions

**The Integrated Breeding Workbench** The workbench starts as a blank slate and the first task for the user is to open or create a project. A project manages a breeding workflow for a particular crop and a specified user. The initial sets of tools which should be available are grouped in seven modules: Administration Tools, Configuration Tools, Query Tools, and Workflow Initialization Tools (genealogy, data management, analysis, and decision support; Fig. 5).

The administration module of the workbench specifies the crop, which identifies the central (public) data resources that will be accessible to the project. This includes a central genealogy database, a central phenotype database, a public gene management database, and a central genotype database. Each installation provides access to local (private) data resources. These data resources include a private or local database for the above data types as well as a seed inventory management system. Each installation has at least one user with administrative privileges. Users are identified by authentication codes (username and password) for access to specific private data resources. ("Private" simply means "requiring authentication for access" and several users may have access to the same private data.)




**Molecular Breeding Platforms in World Agriculture. Figure 4**
The IBP configurable workflow system




**Molecular Breeding Platforms in World Agriculture. Figure 5**
The integrated breeding workbench

The first functionality of the workbench asks the user to open a project by selecting from a list of available project configuration "files." Once the configuration is selected, the availability of the public data resources should be checked, the user authentication codes verified, and the local data resources checked. Next, the list of modules should be reviewed and checked for availability and, depending on the state of the workflow, icons or menus should be made available for modules and tools.

The configuration tools allow users to:

- Select or specify naming conventions for germplasm, germplasm lists, studies, etc.
- Use and update ontologies such as germplasm methods and the trait dictionary
- Update breeding, testing, or collection locations
- Create and modify study templates

The query tools will depend on the data resources specified in the project configuration, and examples are:

- A germplasm and pedigree viewer
- A study browser to view phenotype or genotype data
- A data miner for identifying data patterns
- A cross-study query builder for linking different data sets
- A gene catalog viewer for viewing genetic diversity
- A genotype and trait viewer for visualizing graphical genotypes and trait markers

The workflow initialization tools comprise a set of modules (genealogy, data management, analysis, and decision support tools) that provide the user with a choice of different tools to achieve precise breeding objectives. Users might construct different breeding workflows to match their project activities. The user will only see the workbench tools and settings for those tools required to execute the steps in a particular breeding workflow, and at the appropriate step in that workflow.

The development of each tool is overseen by a team of IBP researchers, developers, and users who design, mock up, and prototype the tools of the breeding application and pass the specifications to a software engineering team. They will then monitor the development and test and support the application. For each application, the team develops a description of the application, functional specifications of all the tools, workflow specifications for the application, and an interface mockup. A workflow for a MARS project is shown in Fig. 6.

**Component 3: IBP Services**

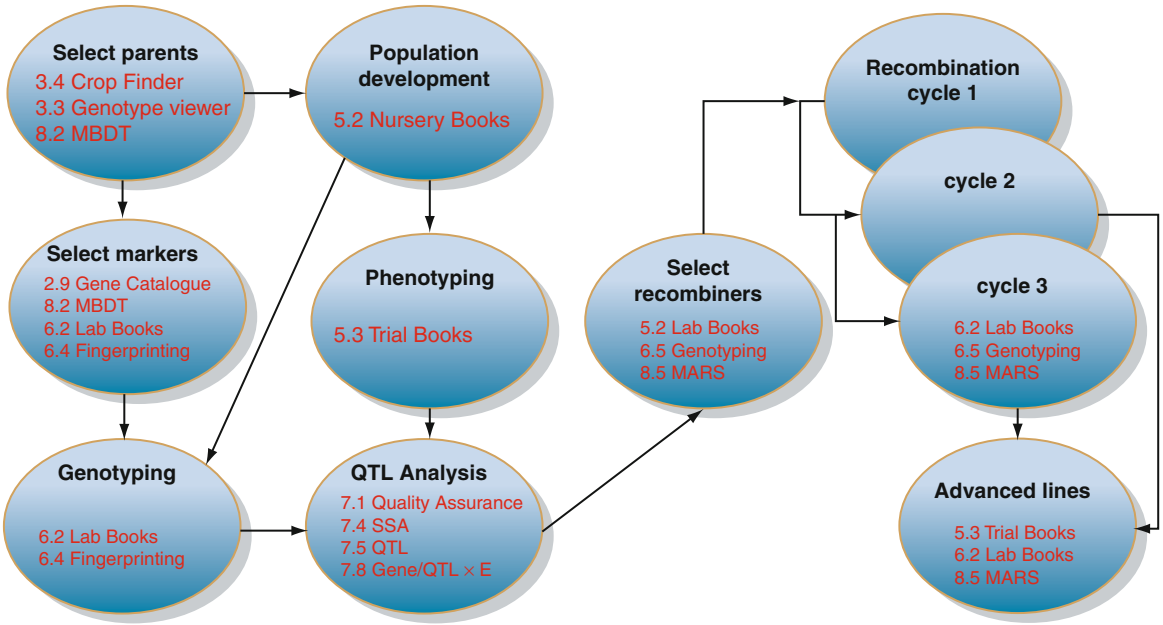
The Services component comprises two modules. The first module, Breeding Services, provides services to conduct MB projects. The second module, Support Services, deals with training and capacity-building, aiming to provide support and improve capacity of NARS breeders to deliver improved germplasm through marker approaches – essential for the adoption of MB approaches and the MBP.

**Breeding Services** These services provide access to specific germplasm, and assist with contracting a service laboratory to conduct the marker work or to quantify specific traits, such as metabolite profiles or grain quality parameters. The module has three elements (Fig. 7):

Genetic Resource Support Service: Access to suitable germplasm and related information from the different partners is a critical element of the portal. To address this, a Genetic Resource Support Service (GRSS) plans to tap into the CGIAR System-wide Genetic Resources Program (SGRP), a collaborative effort between GCP and existing gene banks in the CGIAR and NARS. The GRSS should ensure quality control, maintenance, and distribution of genetic resources, including reference sets and segregating populations acquired or generated through projects supported by GCP, and material generated from other sources and deposited with the GRSS (e.g., maize introgression lines from Syngenta).

Marker Service: The portal provides a set of online options for users to access different high-throughput marker service laboratories in the public and private sectors with clear contractual conditions. Service Laboratories have been selected on the basis of competitive cost, compliance with quality control requirements, and expeditious delivery, but are currently accessible by offline processes pending deployment of the IBP portal.

Trait and Metabolite Service: The portal provides a set of options for users to access laboratories




**Molecular Breeding Platforms in World Agriculture. Figure 6**
Breeding workflow for an MARS experiment

specialized in the evaluation and analysis of specific traits, such as quality traits, pathology screening, or metabolite quantification. Analyses of certain secondary traits and metabolites that are indicative of plant stress tolerance can potentially provide valuable information to be used in breeding. Such analyses are generally prohibitively expensive if done locally, as it is difficult to maintain assay quality and devote the necessary resources for expertise, quality control, and specialized facilities.

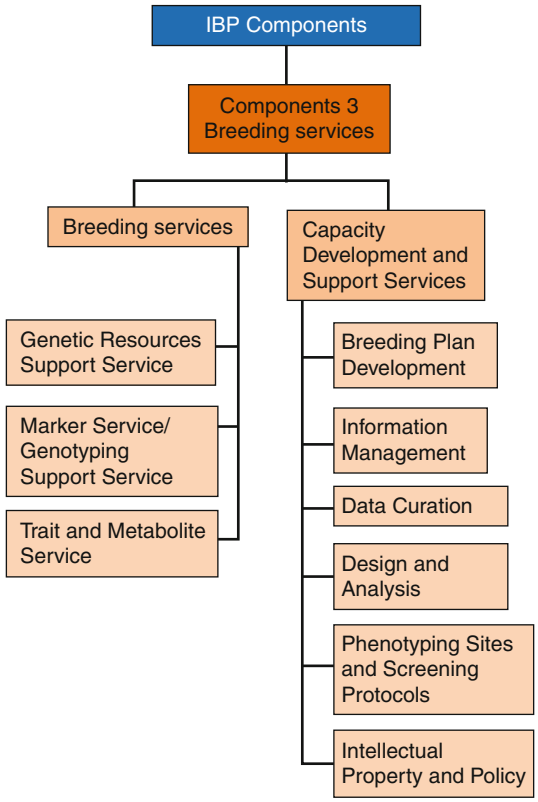
**Capacity Development and Support Services**
Capacity development is an integral part of the project, encompassing training and support in using MB techniques and markers, designing breeding strategies, quality data management, information analysis and decision modeling, phenotyping protocols, and protection of intellectual property (IP).

The main objective of this set of services is therefore to provide backstopping and training in a broad set of disciplines, to complement the elements of the breeding services and address specific technical and logistical bottlenecks. Such expert assistance is essential for the adoption and proper use of new technologies. Services that will be available include:

Breeding plan development: It is essential to develop a breeding plan with a cost–benefit analysis before conducting a multi-cycle MB project. Depending on the nature of the experiment, such a plan may be quite simple or very elaborate, from the transfer of a single region (e.g., transgene) to complex selection that can consider the simultaneous transfer of dozens of regions. The critical factor is that the plan must detail all the activities over time, and the costs and benefits of the project to determine if it is worthwhile conducting the experiment. The platform provides templates and associated cost calculation sheets for different breeding schemes.

Information management: Under this service, assistance is provided in installing and parameterizing the platform IS for use by specific breeding projects.

Data curation: This service assists with capturing and curating current data for particular breeding projects, and in entering them into the integrated IS. This step is absolutely critical for quality control and further sharing of the information, and a contact person for each of the pioneer user cases has been identified to ensure good communication between the platform and the users.




**Molecular Breeding Platforms in World Agriculture.**
**Figure 7**
Organogram of the services provided by the IBP

Design and analysis: This service provides support on statistics, bioinformatics, quantitative genetics, and molecular biology. It includes training in data generation, handling, processing, and interpretation, as well as experimental design from field planting to MAS and MABC schemes. It provides assistance with the "translation" of the molecular context to the breeding context, and it will ensure that the methodology developed for the design and analysis of breeding trials is rapidly available to the users.

Phenotyping sites and screening protocols: Through this service, users can access information on phenotyping sites, protocols, and potential collaborators to ensure that selection is carried out under appropriate biotic and abiotic stresses and that the adaptation of germplasm is well characterized. Characterization of phenotypic sites includes geographical information, meteorological historical data, soil composition, and field infrastructure.

Genotyping Support Service (GSS): The GSS aims to facilitate access by developing country national agricultural research institutes to genotyping technologies, and bridge the gap between lab and field research. This service provides financial and technical support for NARS breeders to access cost-efficient genotyping services worldwide and supports training activities in experimental design and data analysis for MB projects.

Intellectual property (IP) and policy: This service provides support on IP rights and freedom to operate in the arena of biotechnology and germplasm use. The service is currently being provided on an experimental basis through a virtual IP Helpdesk hosted by the GCP web site at http://www.generationcp.org/iphelpdesk.php.

## Integrated Breeding Hubs

If today few question the usefulness of local basic laboratories, it is also generally accepted that large-scale genotyping activities are best outsourced to cost-effective, high-throughput service laboratories, irrespective of location. Following that rationale, the IBP provides access to marker service laboratories as the main avenue to generate the large amount of genotyping data that will be necessary to support the extensive MABC programs of the future, starting with the user cases, but the GCP also recognizes the need to provide breeders in developing countries with access to some regional hubs. At the beginning of the project four regional hubs are envisioned, covering the needs of the Americas – Centro Internacional de Agricultura Tropical (CIAT, www.ciat.cigiar.org); Africa – BioSciences eastern and central Africa (BecA, http://hub.africabiosciences.org); South Asia – International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, www.icrisat.org); and South East Asia – International Rice Research Institute (IRRI, www.irri.org).

These regional hubs are expected to provide the following services:

- In-house hands-on training (different formats are possible from short- to medium-length periods), with the objective of exposing scientists to new technologies and their applications to breeding.
- Training courses for selected groups of researchers, targeting basic knowledge of marker technologies and their applications, as well as data analysis.

These courses can be used for the testing and validation of learning materials, which will then be continuously upgraded.

- Facilitation of small genomic and genotyping projects led by national programs, academia, and small and medium enterprises (SMEs).
- Marker services for "small" and "orphan" crops that do not have mass demand from breeding programs and would therefore not benefit from large service providers, due to the lack of availability of SNP markers and the need to use lower-throughput SSR or other markers that can more easily be handled in lower-tech laboratories.

The Genomics and Molecular Breeding Hubs should help raise the visibility of the IBP and thus help promote the adoption of MB. Collaboration between the IBP and the regional hubs is anticipated to occur through sharing information, guiding users to apply for the appropriate service, organizing training events, and planning other developments of common interest.

## Scope and Potential for Molecular Breeding Platforms

### Gaps Across Countries and Crops

The application of MB approaches is now routine in developed countries, as is the integration of facilitative information and communication technologies, which are critical given the immense volumes of data necessary for, and generated by, these breeding processes. However, the situation is very different in developing countries, where MB is still far from routine in its application in breeding programs, particularly in Africa. This is especially critical due to the monumental and urgent imperative to rapidly achieve food security and improve livelihoods for a rapidly growing population through breeding for biotic stresses (including weeds, pests, and diseases) and abiotic stresses (including physical soil degradation, nitrogen deficiency, drought, heat, cold, and salinity) – conditions that make accurate phenotyping challenging. Fortunately, the history of modern breeding in developing countries is comparatively short, allowing a larger potential for crop improvement relative to the genetic gains that can be obtained at this time in developed countries, in which extensive breeding has been applied to crops for a longer time.

To address these issues, the capacity of national research institutions in terms of funds, infrastructure and expertise is directly related to the strength of their national economies [86]. This is reflected in the sharp differences in the capacity to conduct and apply biotechnology research as observed across developing countries (FAOBioDeC, http://www.fao.org/biotech/inventory_admin/dep/default.asp), and by the same token in their capacity to establish and/or utilize MBPs. The result is a three-tier typology of developing countries, directly attributable to the level of each country's investment in agricultural R&D [87].

Tier-1 countries, comprising newly industrialized countries (NICs) such as Brazil, China, India, Mexico, South Africa, and Thailand, substantially invest in technology and R&D and are self-reliant in most aspects of marker technologies [88, 89]. These countries have the simultaneous potential to effectively adopt, adapt, and apply information and communication technologies to enhance research efficiency and outputs. They are therefore naturally at the vanguard in adopting MBPs.

Mid-level developing world economies (tier-2) such as Colombia, Indonesia, Kenya, Morocco, Uruguay, and Vietnam are well aware of MB's importance, and some effectively apply marker technologies for germplasm characterization [90–93] and selection of major genes [94–99]. These countries have a matching potential for a limited utilization of MBPs, a potential that can be enhanced fairly rapidly in the medium to long term.

Low-level developing world economies (tier-3 countries) are struggling to sustain even basic conventional breeding. They have very limited or no application of MB approaches and are unlikely to adopt MBPs except in the long term.

Especially for tier-3 countries, resource-limited breeding programs in many developing countries are severely hampered by a shortage of well-trained personnel, low level of research funding, inadequate access to high-throughput genotyping capacity, poor and inadequate phenotyping infrastructure, lack of ISs and appropriate analysis tools, and by the logistical difficulty of integrating new approaches with traditional breeding methodologies – including problems of scale when scaling up from small to large breeding programs.

Until recently, the scarcity of available genomic resources for clonally propagated crops, for some neglected cereals such as millet, and for less-studied crops such as most tropical legumes, which are all very important crops in developing countries, represented a further constraint to agricultural research for development [100], thereby limiting the application of molecular approaches and hence the potential for MBPs. However, the recent emergence of affordable large-scale marker technologies (e.g., DArT [101]), the sharp decline of sequencing costs boosting marker development based on sequence information [102], and the explicit efforts of national agricultural research programs (e.g., India [103]) and international initiatives such as GCP [104]) have all resulted in a significant increase in the number of genomic resources available for less-studied crops. As a result, most key crops in developing countries now have adequate genomic resources for meaningful genetic studies and most MB applications.

Similarly, international efforts such as GCP's IBP are designed to help overcome the challenges of developing-country breeders – exploiting economies of scale by making available convenient and cost-effective collective access to cutting-edge breeding technologies and informatics hitherto unavailable to them, including genomic resources, advanced laboratory services, and robust analytical and data management tools. Together, this increasing availability of genomic resources and tools for previously neglected but important crops and the access to initiatives targeting the resource-challenged NARS of the developing world will hasten the adoption of MBPs for these countries.

### Institutional, Governmental, and Public Support

While corporate and other proprietary MBPs need only meet the specific requirements of a particular corporation or of specific paying clients, the development of platforms targeted at breeding programs in the developing world require a broad consensus among the parties that would use them and support them from multiple overseeing organizations. This is because these platforms are built on the premise of minimizing costs and maximizing benefits through economies of scale generated through collective access by multiple partners.

The public-access MBPs would therefore be critically dependent on well-structured MB programs, which may not be a reality in many developing countries. A good structure would entail compliance with common or compatible:

- Good field infrastructure, including meteo station
- Good agronomical practices at experimental stations
- Crop ontology information system
- Data collection, management, and analysis protocols
- Breeding plan design
- Information and communication technology infrastructure
- Informatics tools for analysis, decision support purposes, and eventually modeling and simulation

Traditionally, developing world breeding programs have largely been poorly funded and poorly supported, and have been primarily driven by donor organizations [105, 106]. The lack of in-country support has often limited the dependent breeding activities to no more than a basic level. Under such circumstances, it was unrealistic to anticipate the adoption of new biotechnologies – including the utilization of MBPs. Fortunately, this scenario is changing. In 2003, through the Comprehensive Africa Agriculture Development Programme (CAADP, http://www.caadp.net/implementingcaadp-agenda.php), African governments committed to invest more in food security and in agriculture-led growth. Since then, many countries in Africa and elsewhere have developed comprehensive agricultural development strategies.

There is also a growing participation by foundations and nongovernmental organizations, and more recently the emergence of public–private sector partnerships (e.g., US Global Food Security Plan, http://www.state.gov/s/globalfoodsecurity/129952.htm). This governmental and institutional commitment is critical for the adoption of biotechnologies in general [8, 107] and for MB adoption in tier-2 countries in particular, with the attendant establishment and utilization of MBPs.

### Challenges, Risks, and Opportunities

Challenges hampering the potential of MBPs in developing countries include both factors applicable generally to MB and those specific to MBPs. These

factors encompass infrastructure capacity, human resource, and operational and policy issues. But amidst the challenges there are also actual and potential opportunities.

**Human Capacity** Human capacity for MB technologies in developing countries is a challenge, and limitations include substandard agriculture programs at universities; difficulties in keeping up to date with relevant developments, including failures by others; poor technical skills in core disciplines; isolation as a result of insufficient peer critical mass in the workplace; and poor incentives to attract and retain scientists, resulting in brain drain and staff turnover [108].

To partially offset the undesirable trend of losing the "champions" and to "generate" more "champions," novel international initiatives like Alliance for a Green Revolution in Africa (AGRA) support high-quality education in the South. Examples include the African Centre for Crop Improvement (ACCI, http://www.acci.org.za/) based at the University of KwaZulu–Natal in South Africa and the University of Ghana-based West African Centre for Crop Improvement (WACCI, http://www.wacci.edu.gh/). Both institutes offer doctorate degrees in modern breeding to African students, with the fieldwork component being carried out in the students' home countries.

While obtaining their Ph.D. in plant breeding, these scientists study the principles of marker technologies, equipping them to undertake MB activities. To retain this much-needed expertise in Africa, the WACCI and ACCI programs also provide post-Ph.D. funds for these scientists to conduct research in their home countries and, in some cases, provide matching funds for their career advancement.

**Precise Phenotyping** There can be no successful MB program without precise phenotyping of the target traits. Reliable phenotypic data is a must for good genetic studies [109] and most developing countries lack suitable field infrastructure for good trials and collection of accurate phenotypic data. As part of the services of a good MBP, guidelines on best practice must be provided on how to design and run a trial and conduct precise phenotyping for genetic studies under different target environments. Improving access to homogeneous field areas, and paying attention to good soil preparation and homogeneous sowing are critical. The development of new geographic IS tools [102, 110], experimental designs, phenotyping methodologies [111, 112], and advanced statistical methods [113] will facilitate the understanding of the genetic basis of complex traits [114] and of genotype-by-environment (G×E) interactions [48, 115]. Improving phenotyping infrastructure in developing countries must thus be a top priority to promote modern breeding and utilization of MBPs [106].

**Laboratories for Markers Services** Genotyping can be expensive when it is performed in small laboratories using labor-intensive and low-throughput markers such as SSRs. This has traditionally limited the use of MMs in developing countries beyond the fingerprinting of germplasm with a small number of markers or the use of MAS for a few key traits. Operational efficiency is also vital, because fundamental timelines must be respected to ensure that no crop cycle is lost. Indeed, at every selection cycle, a service laboratory may have only a few weeks (time between DNA being extracted from leaves harvested on plantlets and the flowering time) to conduct the analysis and return the data to the breeders to enable them to conduct appropriate crosses among selected genotypes.

There is general agreement today that basic local laboratories at national and regional levels can be useful at least to service small local needs such as fingerprinting of limited number of accessions, GMO detection or MAS for specific traits, or for teaching and training purposes. It is also generally accepted that large-scale genotyping activities are best outsourced to advanced, modern, cost-effective high-throughput service laboratories, irrespective of the original location of the needs. This outsourcing is driven by the evolution in marker technologies. The advent of SNP genotyping led the shift from the low-throughput, primarily manual world of SSRs to high-throughput platforms powered by robotics and automated scoring, better handled by dedicated service laboratories [102, 116, 117]. As a result, genotyping costs have decreased by up to tenfold while data throughput has increased by the same magnitude. An example for MARS is provided in Fig. 6. SNP markers are increasingly available for most mainstream crops

and for several less-studied crops [118, 119], which are important in developing countries.

A particular effort will be needed to ensure an easy and reliable way to track samples from the field to the laboratory, and back to the field – it will hence be vital to carefully identify DNA samples from material collected in the field. Such documentation should optimally be through bar-coding, and all information pertaining to management of field trials or experiments should be recorded in electronic field books. Marker work would of necessity be subcontracted to a service lab with a good and preferably platform-compatible laboratory information management system (LIMS).

**Data Management** For breeders to efficiently access relevant information generated by themselves and by other researchers, reliable data management (including sample tracking, data collection and storage, and modern analytical methodologies and tools for accurate decision making, among others) is critical both within a given MB program and across programs. In view of this, it is essential that breeders manage pedigree, phenotypic, and genotypic information through common or mutually compatible crop databases, in keeping with the collective access principle of a public MBP. The format of databases would need to be user-friendly and compatible with field data collection devices and applications to encourage both adoption and compliance. Ultimately, data collection and management processes would need to seamlessly link with a platform-resident analysis, modeling, simulation, and a decision support workbench for full utility of the breeding platform.

**Paradigm Shift: Collaborative Work and Data Sharing** Access to information and products generated by fellow users is a potentially critical incentive for breeders to use the platform and share their own data with other users. However, this would require a fundamental paradigm shift from the present data-hoarding, inward-looking approach to research common to breeders. This may, however, only be achievable if it is a clear requirement in the terms of engagement for membership of a "platform community," or if distinct financial and other incentives are offered for such sharing.

**Technology-Push Versus Demand-Driven** An MBP is by nature a high-level technological solution. It carries with it the inherent risk of failing to address fundamental practical problems of developing-world breeding programs, which will often by nature be technology-deficient. Such platforms therefore face the challenge of ensuring that they meet targeted user objectives and address practical constraints.

However, with this challenge comes an opportunity to introduce advanced MB methodologies to developing world breeders, by encouraging change that will enable them to take advantage of the efficiencies and economies of scale offered by the MBP. This opportunity would be particularly reachable with bottom-up platform design and development that actively engages and involves the breeders – including elements of human resource capacity development and support in usage.

**Adoption and Use by Breeders** An MBP would only make a difference if it is adopted and widely used by the breeders. The most important element influencing this would be credibility – a function of the quality of the technology, the awareness of potential users, the ease of access, and initial incentives. There is a need for successful public sector developing-country examples to demonstrate that the platform can effectively enhance the efficiency of breeders through the use of modern approaches – a clear demonstration of the added value of using the platform.

**Sustainability of the Platform** Sustainability would be a challenge for MBPs targeting developing world breeding programs, given their resource limitations. These programs may not be able to meet the full cost of platform usage, and the cost of maintaining and updating the different elements of the platform on a regular basis – particularly tools and facilities that must keep abreast with evolving information and communication technologies.

Of course, platform sustainability is directly linked to its adoption by breeders, and sustainability strategies must be adapted to the diversity and financial resources of the potential clients, from developing-world national agricultural research institutes with limited resources to SMEs. Service costs might also be adjusted

if clients are willing to share data and release germplasm through the platform.

Platform managers may also have to consider other innovative options like on-platform advertising by agriculture-related commercial enterprises. However, ongoing donor support would most likely still be required in the medium to long term.

**Communities of Practice** The development of platform-based MB communities of practice, to connect groups of crop researchers, mainly breeders, willing to share experiences and information on modern breeding methods, best field practices, and development of improved varieties, and to practice peer-to-peer mentoring, are an additional potential avenue for platform adoption and sustainability, besides providing means to quickly and efficiently resolve recurring breeding problems. Partnerships between developed and developing-country institutions, and between the private and public sectors, are also an opportunity for realizing the full potential of MB [87, 108].

Many other hurdles limit successful public sector utilization of MB opportunities [120, 121]. However, the potential of virtual MBPs made possible by the revolution in information and communication technologies provides opportunities to counter and overcome many of those shortcomings.

## Potential Economic Impact of Molecular Breeding Platforms

By its nature, MB improves the efficiency of crop breeding – progressively increasing genetic gains by selecting and stacking favorable alleles at target loci. The utilization of MBPs accelerates and amplifies the advantages of MB by introducing significant efficiencies in resource and time usage. Predictive or designer breeding, which would be the ultimate result of information-rich MB, attainable through the use of MBPs by numerous different breeding programs that freely share data and germplasm, would particularly bring about these savings in resources and time.

However, a direct comparison of the cost-effectiveness of MB with phenotypic selection is not straightforward. Firstly, factors other than cost – such as trade-offs between time and money – play an important role in determining the selection method. Secondly, this choice is further complicated by the fact that the two methods are rarely mutually exclusive or direct substitutes for each other [122]. On the contrary, under most breeding schemes, they are in fact complementary. Where operating capital is not a limitation, MB maximizes the net present value, especially when strengthened through MBPs [123]. With the increasing ease of accessing marker service laboratories and the declining cost per marker data point, MB costs are shrinking, making it extremely attractive from a purely economic perspective.

However, once the technological hurdles are overcome, the ultimate impact of new technologies (such as MBPs) is often limited by the lack of, or ineffective, seed distribution systems or by distant markets. SMEs are critical in promoting access to, and distribution of, improved seeds, thus helping alleviate a major bottleneck to the impact of improved breeding on smallholder farmers [124, 125].

Few economic analyses have been conducted to objectively assess the potential impacts of MB in the public sector, and none for MBPs that are just now emerging as a tool for breeding in the public sector.

Of the few analyses done to date, one evaluates the economic benefits of MABC using preexisting MMs in developing rice varieties tolerant to salinity and P-deficiency [126] in Bangladesh, India, Indonesia, and the Philippines. Encompassing a broad set of economic parameters, the study concluded that MABC saves an estimated minimum of 2–3 years, resulting in significant incremental benefits in the range of USD 300–800 million depending on the country, the extent of abiotic stress encountered, and the lag for conventional breeding [127].

Future studies are likely to confirm the positive economic benefits of MB and, given that MBPs amplify the benefits of MB, it can be reasonably inferred that the emerging platforms would indeed further enhance those economic benefits.

## Future Directions

MBPs will inevitably have a significant impact on crop breeding in developing countries in the medium to long term because of:

- The needs-driven demand for improved crop varieties to counter the global food crisis

- The exponential development of genomic resources
- The ever-declining cost of marker technologies
- The increasing occurrence of public–private partnerships, where the public sector can learn from private companies about best practices for integrating MB into their breeding programs
- The need for innovative solutions to the challenges of resource and operational limitations

The first challenge of MBPs will be to meet the immediate needs of the breeders in developing-country public and private programs. The first step will be to provide them with the tools for enhancement of their current breeding programs, through the implementation of field books, pedigree management, and basic statistical analytical tools necessary to optimally conduct their current breeding efforts. In close succession with these first applications, tools will need to be made available to facilitate the integration of MB into their breeding programs. Databases will need to be developed for storing genotypic and phenotypic data, integrated analytical tools will need to be made available to breeders for analysis of this accumulated data and for the identification of important simple trait loci or QTLs to monitor and recombine in their breeding programs, and decision support tools will need to be developed to help breeders decide on the next steps to engage in based on the data they generated from their MB activities.

In the near future, more complex tools will need to be developed for the storage and analysis of the large amounts of genotypic data that will be generated by new next-generation sequencing technologies and for their application in GWS. A tight linkage will also have to be established with the wealth of information that is being generated and will continue to be generated even faster in the genomics area, leading to the dissection of the genome and to the discovery of the location and function of major genes having an impact upon the performance of crops in environments relevant to developing-country programs.

Eventually, the accumulation of large amounts of genetic information linked to specific haplotypes will lead to the increasing use of predictive breeding in combination with traditional MB usage and appropriate tools will also need to be developed to support those efforts.

Although it is critical for a platform to anticipate all the new possible features of MB, ensuring that new technologies and ISs will find their way in a flexible infrastructure, it is also quite probable that most of the breeding programs in developing countries will work at the short- and mid-term mainly with simple MB approaches as they will never reach the critical size of crosses and germplasm evaluation requested to maximize complex approaches.

## Conclusion and Prospective Scenarios

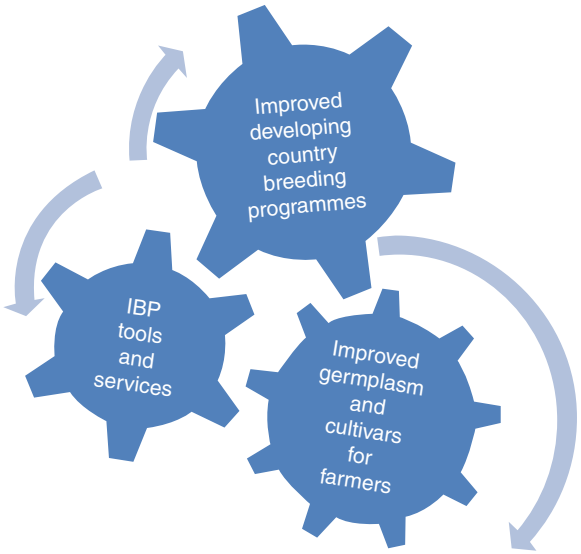
Through international initiatives like the ones coordinated by the CGIAR centers and programs, several notable developing-world MB successes have already been reported.

A well-known example is the development of submergence-tolerant rice cultivars through MABC led by IRRI [128]. The introgression of the Sub1 gene from FR13A (the world's most flood-tolerant variety) into widely grown varieties like Swarna improved yields in more than 15 million hectares of rain-fed lowland rice in South and Southeast Asia.

MB in general and the use of MBPs in particular have definitely been shown to be an efficient approach for reducing the number of required selection cycles and for increasing the genetic gain per crop cycle to a point where the required human and operational resources can be kept to a minimum.

However, for sustainable adoption, the use of modern breeding strategies requires a breeder-led bottom-up approach. As a start, simple MB approaches adapted to local environments should be tested first by individual breeders to evaluate their success and impact under those breeders' conditions. Once proven, these approaches can then be implemented more widely or integrated to an MBP for enhanced efficiency. In case of individual success the adoption of MB by those breeders should be quite straightforward.

It is clear that the extent, speed, and scope of adoption of MB approaches and of utilization of MBPs will vary somewhat across tier-1, tier-2, and tier-3 countries, depending on the local priorities and on the resources available in given breeding programs. It is unrealistic to expect that large-scale MB breeding activities, including utilization of MBPs, will be widely implemented across the board in developing




**Molecular Breeding Platforms in World Agriculture. Figure 8**
IBP as a key component to boost NARS breeding capacities and therefore crop productivity in developing countries

countries in the near term. However, the prospects are bright for individual breeders in these countries (particularly in tiers 1 and 2) to access germplasm, data, tools, and methodology that will allow them to conduct efficient MB projects by taking advantage of large international initiatives specifically targeting developing-country breeding programs. This will, however, happen in different ways and on different timelines for each tier.

For tier-1 countries, the impact would be evident in the shorter term – say in 3–6 years. These countries will benefit from new tools and platforms by increasing the rate of MB adoption. The biggest change is likely to occur in tier-2 countries, as these countries would be starting MB from scratch, but the impact would realistically be measurable only in the medium term, meaning in about a decade from now. For countries currently in tier-3 to advance to tier-2, basic breeding programs must first be established, which is highly dependent on governmental priorities and on subsequent resource allocation.

All in all, implementing MB (and catalyzing and accelerating its impact through MBPs) will boost crop production, which will translate into higher farm productivity per unit of land, better nutrition, higher incomes, poverty alleviation, and ultimately improved livelihoods in developing countries (Fig. 8). These gains will be amplified by sustained use, by continuously improving expertise, and by growth and development of homegrown capacity for the application of advanced breeding approaches.

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# Mussel Culture, Open Ocean Innovations

Richard Langan
Atlantic Mariner Aquaculture Center, University of New Hampshire, Durham, NH, USA

## Article Outline



## Glossary

**Suspension culture** A production method for mussels and other shellfish that employs ropes, cages, or nets suspended in the water column from either rafts or longlines.

**Surface longline** An anchored structure consisting of surface floatation supporting one or more horizontal lines from which ropes, cages, or nets can be suspended in the water column.

**Open ocean farming** Refers to aquaculture production of marine organisms in open ocean or offshore waters that are removed from any significant influence of land masses.

**Submerged longline** Subsurface structure consisting of anchors and submerged floatation from which ropes, cages, or nets can be suspended.

**Site selection** The process for selecting farming sites based on specified parameters such as depth, current and wave climate, temperature, and primary productivity.

**Environmental effects** The effects of farming activities on the physical, biological, and chemical properties of the marine environment *and* the effects of the environment on cultured organisms and consumers of cultured food products.

**Seston** Particulate material suspended in the water column of water bodies consisting of both living and dead organic material and inorganic particles.

**Pseudofeces** Suspended particles that have been rejected as food by filter feeding bivalve mollusks. The rejected particles are wrapped in mucus and expelled without being passed through the digestive tract.

## Definition of the Subject

Aquaculture production of several species of mussels in sheltered marine waters is well established and occurs in many countries worldwide. The primary method of production of high quality mussels is suspension of ropes with attached mussels from floating rafts or surface longlines that are anchored to the seafloor. While demand for fresh, frozen, and canned mussel products continues to increase, growth in production is hampered by a lack of suitable space for expansion in sheltered waters. For more than a decade, there has been interest in developing production methods suitable for open ocean environments where wind and wave conditions preclude the use of either rafts or surface longlines. Recent advances in the use of longlines that can be submerged below the sea surface and therefore avoid the upper portion of the water column that is most affected by wave energy indicate that open ocean production is feasible. However, additional development in technology and methods to improve production efficiency and insure worker safety, as well as changes to political and regulatory frameworks are needed in order to achieve large-scale production.

## Introduction

Population growth and consumer preference have resulted in a growing demand for seafood, a trend that is projected to continue into the future [1]. Production from capture fisheries has leveled off, and by most projections will remain stagnant or decline, depending on management and regulatory measures implemented by fishing nations [2, 3]. In contrast,

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aquaculture production has increased by nearly 10% each year since 1980, and has played an important role in filling the gap between seafood supply and demand. Only a few decades ago, wild-caught fish and shellfish supplied nearly all edible seafood, though with essentially flat growth since 1980 and the rise of aquaculture over the same time period, capture fishing now accounts for only about half of the total [1]. In the most optimistic scenarios, wild-caught fisheries production will remain stagnant [2]; therefore, growth in the global seafood supply will continue to rely on aquaculture production.

There are signs, however, that the rate of growth for global aquaculture may have peaked for land-based and nearshore marine culture due to political, environmental, economic, and resource constraints [1]. Expansion of land-based culture is limited primarily by economics, particularly in developed countries where costs associated with land, capital equipment, and energy required to pump and treat water are prohibitive. In addition, very few marine species are appropriate for land-based culture. For example, the space and volume of phytoplankton required to produce large quantities of filter feeding mollusks in land-based systems would be enormous, and therefore not economically viable.

For nearshore marine farming, available and suitable space is the primary limiting factor as sheltered coastal waters are for most countries quite constrained to begin with and are already used for a multitude of commercial and recreational activities with which aquaculture must compete for space [4]. Expansion of large-scale finfish farming in coastal waters is also limited by environmental concerns. While there are also concerns about potential environmental effects of bivalve mollusk culture, they are minor in comparison to net pen culture of finfish and are balanced by recognition of the ecosystem services such as enhanced habitat complexity and filtration capacity provided by mollusks [5]. It is rather the effect of environmental conditions on mollusk culture, and specifically the effects of pollution on product safety that is limiting expansion in nearshore waters. Rapid coastal development and population growth and the resulting increase in human sources of pollution have affected the sanitary quality of nearshore waters, rendering shellfish grown there unsafe for consumption. As a consequence, many otherwise suitable sheltered sites for mollusk culture are off limits due to public health restrictions.

In developed countries, conflict with coastal residents and tourist-related businesses over aesthetic values, primarily over water views from shorefront property, have also affected the establishment of new farming sites. As the demographic of coastal communities continues to change and new residents place more value on views and recreation than food production, these conflicts are likely to increase. Given the constraints on expansion of current methods of production, it is clear that alternative approaches are needed in order for the marine aquaculture sector to make a meaningful contribution to the world's seafood supply.

Farming in open ocean waters has been identified as one potential option for increasing production and has been a focus of international attention for more than a decade. Despite the global interest in open ocean farming, development to date has been measured, primarily due to the significant technical and operational challenges posed by wind and wave conditions in most of the world's oceans [4]. Farming in fully exposed open ocean waters requires a different engineering approach since equipment and methods currently used in sheltered nearshore sites are largely unsuitable for the open ocean. In addition, the scale of investment required to develop and demonstrate new technologies and methods for offshore farming is yet to be determined, though most engaged in this endeavor would agree that it will likely be substantial.

Despite these challenges, there is sufficient rationale for pursuing the development of open ocean farming. Favorable features of open ocean waters include ample space for expansion, tremendous carrying capacity, less conflict with many user groups, reduced exposure to human sources of pollution, the potential to moderate some of the negative environmental and aesthetic impacts of high density coastal farming [6–8], and optimal environmental conditions for some bivalve mollusk species [9, 10]. For many countries, where cost, environmental concerns, limited space, and competing uses have restricted growth of land-based and nearshore marine farming, few other options for significant expansion exist.

Of the many species of finfish and shellfish that have been considered for open ocean farming, several

species of mussels have emerged as attractive candidates. There are several reasons for this. Like all filter-feeding mollusks, mussels derive all their nutritional needs from naturally occurring phytoplankton and organic particulates. Therefore, daily visits to deliver formulated feed by service vessels and farm personnel, which may be prohibited for extended periods by sea conditions, are not needed, nor is on-site infrastructure for automated feeding, which is both costly and vulnerable to damage from storms. Unlike many cultured species that have gradually transitioned from wild capture to aquaculture, farming has been the primary means of production for mussels for many decades; therefore, methods used in sheltered waters are well developed, highly automated, and very efficient [11]. Mussels are also relatively fast growers, with production cycles ranging from 12 to 18 months [9, 12].

Production methods in sheltered nearshore waters include bottom culture, which is practiced in some locations such as the Netherlands, Scandinavia, and the USA (Maine), and pole or "bouchot" culture, which is practiced in France; however, suspension culture, because of superior product quality, accelerated growth, and opportunities for mechanization, has emerged as the leading method of production [11]. Techniques and materials used for suspension culture may vary somewhat from place to place; however, in general, culture methodology consists of suspending mussel ropes or "droppers" from either rafts or longlines [13]. Raft culture was pioneered in Spain and from there became established in Scotland and more recently in Maine USA and in the Pacific Northwest coast of North America [11]. While rafts can be highly productive, they are suitable for use only in very sheltered embayments. Longline technology, which was developed in Japan, consists of either surface or submerged longlines, held in place with anchors and supported by buoys or floats. As with raft culture, surface longlines are only suitable for use in sheltered waters [13]; therefore, in locations where adverse sea conditions or drift ice occur, submerged longlines are the only option. Submerged longlines have been used primarily in locations (e.g., Atlantic Canada) where winter ice would impact buoys and lines [14]. It is only in recent years that the technology has been used in fully exposed open ocean locations [9].

## Characterization and Selection of Open Ocean Farming Sites

Before discussing approaches to the development of open ocean mussel culture, it is important to first define what is meant by the term "open ocean." For most engaged in this sector, it is used synonymously with "offshore" and is generally accepted to mean farming in locations that are subjected to ocean waves and currents and removed from any significant influence of land masses rather than a set distance from shore. Clearly, a wide range of sea conditions falls under this broad definition. Ryan [4] reported on a site classification system for marine waters developed in Norway that is based on significant wave height exposure (Table 1).

While this classification method is instructive, knowledge of the full range of conditions at a particular site is needed to develop appropriate technologies and safe and efficient operating procedures.

There are a number of criteria that determine the suitability of open ocean sites for farming, many of which are also considerations for sheltered waters. These include proximity to infrastructure such as ports, processing and distribution centers, as well as physical and biological criteria such as bathymetry, seabed characteristics and contour, current velocities, temperature profiles, dissolved oxygen, turbidity, the quantity of quality of phytoplankton, and the frequency of occurrence of harmful algal blooms. The most important additional feature of offshore sites is wave climate. Significant wave heights, wave periods, the frequency and duration of high energy storm

**Mussel Culture, Open Ocean Innovations. Table 1** Norwegian classification of offshore waters based on significant wave heights (From Ryan [4])

| Site Class | Significant wave height (m) | Degree of exposure |
|---|---|---|
| 1 | <0.5 | Small |
| 2 | 0.5–1.0 | Moderate |
| 3 | 1.0–2.0 | Medium |
| 4 | 2.0–3.0 | High |
| 5 | >3.0 | Extreme |

conditions, and the combined forcing of waves and currents must be known in order to determine whether a site is suitable, accessible by service vessels and personnel with reasonable frequency, and if so, what type of technology is required for farming.

It is imperative that a thorough evaluation of the parameters described above be conducted before proceeding with development of a site for farming. The requirements for data and subsequent analysis can be substantial; however, the use of advanced oceanographic technologies can greatly facilitate this task [8]. Multibeam sonar and three-dimensional visualization can generate a wealth of data on seafloor contours and texture to inform mooring system design and placement. Collection of time intensive data on temperature, salinity, dissolved oxygen, turbidity, and fluorescence can be greatly facilitated by strategic deployment of in situ instrumentation at appropriate depth intervals in the water column. Additional instrumentation should include Acoustic Doppler Current Profilers (ADCP) that can measure and record current velocity and direction throughout the water column, wave sensors that can give precise data on wave height, direction, steepness, and period, and meteorological sensors to measure air temperature and wind speed and direction. Many countries have buoy arrays in coastal waters that can provide long-term data on regional climatology to aid site evaluation; however, collection of site-specific data is critical. Assessment of the potential for the effects of global climate change on critical parameters such as water temperature should also be considered.

The data collection period required for site evaluation will vary, depending on local and regional environmental and meteorological conditions. Good baselines for some parameters can be established in a relatively short time frame (1 year), others such as the frequency, duration, and severity of storms or blooms of toxic algae are less predictable and it may take longer to determine the suitability of a particular site.

While most of the focus on open ocean development has been on cage culture of finfish, there has also been growing interest in offshore culture of bivalve mollusks. Some of the same drivers such as ample space and the opportunity to avoid user conflicts are identical to those for finfish culture, though perhaps more importantly, reduced risk of exposure to human sewage and industrial pollution presents a major advantage of open ocean waters over coastal locations.

There are, however, possible limitations as well as advantages. Open ocean waters in many areas of the world are nutrient deficient, so careful attention must be paid during site selection to the quantity, quality, and seasonality of phytoplankton available to dense arrays of filter feeding mollusks. Macroscale information on primary productivity can be obtained from ocean color satellite data generated by instruments such as Sea-viewing Wide Field-of-view Sensor (SeaWiFS) and Moderate Resolution Imaging Spectroradiometer (MODIS). Site-specific data on concentration and composition can be generated by in situ fluorometry and microscopic analysis of the plankton community. Phytoplankton concentration at different depths is also an important factor, as farmers will wish to maximize the use of vertical space for production in deep ocean waters. The frequency and duration of harmful algal blooms (HABs) is also a critical consideration for offshore mollusk farming. In some locations, blooms of toxic algae originate and persist in offshore waters (e.g., *Alexandrium sp.* In the Gulf of Maine, USA) and can result in extended public health closures with severe economic impact on producers.

In addition to physical, chemical, and biological characteristics of a site, other human uses in the vicinity such as shipping, fishing, and mining must be identified in order to avoid conflicts. Involvement of the appropriate permitting authorities in the early stages of development of an open ocean farming site is also critical [15]. Other factors such as use of the area by marine mammals, proximity to foraging areas of predators (e.g., diving ducks), location of sensitive biological communities, presence of parasitic organisms (e.g., pea crabs, trematodes, and copepods), and sediments contaminated by toxic substances must also be considered [16].

## Technologies for and Methods Open Ocean Mussel Farming

Technologies for open ocean mussel farming are essentially adaptations of suspension culture methods employed in sheltered marine waters. Designs and prototypes for submersible rafts have

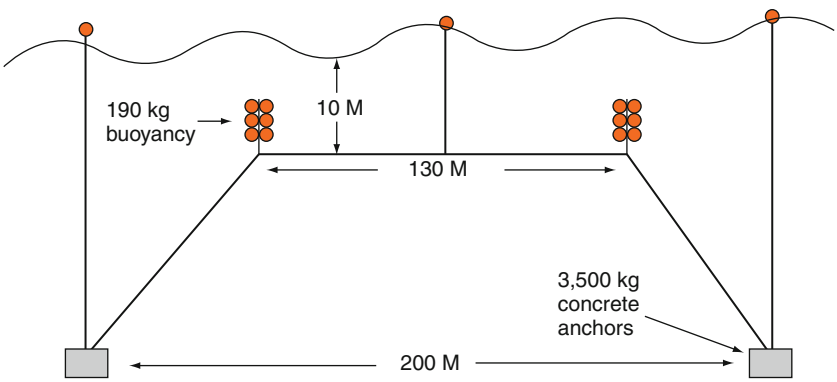
been developed [17, 18]; however, submerged longlines are the most commonly used method. This technology was developed in Japan and has been in use there for several decades for deep water suspended scallop culture, though not in fully exposed open ocean conditions. The technology has been successfully adapted for sheltered water mussel culture in Atlantic Canada where winter and spring drift ice can damage surface longlines [14]. More recently, the technology has been shown to be effective for mussel production in very high-energy open ocean conditions (e.g., significant wave heights >10 m) in the northeast USA [9] and at a test site in the German Bight with significant wave heights >8 m, and current velocity up to 1 $ms^{-1}$ [19]. The technology is quite simple and it consists of relatively inexpensive materials. A design currently in use in North America is presented in Fig. 1.

The structural stability of a submerged longline is maintained by the opposing forces of submerged flotation at the ends of a single horizontal backbone, connected by lines set at a 45° angle to seafloor anchors. The most commonly used anchors are large (3–6 tons) deadweight concrete anchors, though both plow type and screw anchors have been used in some locations. Submergence depth of the backbone is dictated by site-specific wave climate and can range from 3 to 15 m. Surface floatation is minimized to prevent the transfer of wave-induced motion the backbone, and consists of nonstructural marker buoys for the anchor lines and a mid-backbone pick-up line that provides access to the crop from a service vessel. Anchors are generally spaced from 100 to 200 m apart, and depending upon the depth of the water and desired depth of submergence, the backbone length can range from 70 to 130 m. Ropes or "droppers" of mussels are suspended from the backbone, and additional submerged floatation is added as the crop gains mass during growout (Fig. 2).

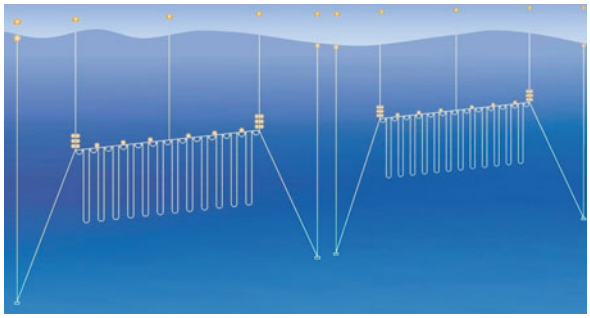
At some of the open ocean farms that have been established, converted fishing vessels are currently used to tend offshore longlines. The deck equipment required for tending lines to seed growout ropes and to inspect and harvest crops is similar to that in use for sheltered sites and includes rail mounted starwheels (Fig. 3) and an articulating crane (Fig. 4).

In addition, equipment common to many fishing vessels such as a lobster or crab trap hauler or a rotating boom is needed for lifting the submerged line to the surface. If there is sufficient deck space, bulk processing equipment such as declumping and debyssing machines can be used during harvest operations to reduce the need for extensive processing at shore-based facilities. Though converted fishing vessels may be used as this sector develops, it is likely that large, seaworthy, specialized vessels that can carry the harvesting and primary processing gear, provide a stable platform for lifting operations and a large load capacity for the harvest will be required to support large-scale operations. Vessels of this nature are in use in France and New Zealand [20].

In addition to submerged longlines, some experimental efforts have employed a submersible ring-like structure attached to a wind turbine tower, which has




**Mussel Culture, Open Ocean Innovations. Figure 1**
A schematic of a submerged longline used for suspension culture of mollusks in open ocean environments



**Mussel Culture, Open Ocean Innovations. Figure 2**
A diagram of a submerged longlines showing the attachment of mussel growing ropes to the backbone and the placement of floatation added to the backbone as the crop increases in mass during growout (From Langan and Horton [9])

**Mussel Culture, Open Ocean Innovations. Figure 3**
A forward looking view of the starboard side of a service vessel showing the backbone of a submerged longline set into aft (foreground) and forward starwheels. Growing ropes with seed mussels are attached to the backbone for the growout cycle

**Mussel Culture, Open Ocean Innovations. Figure 4**
A hydraulic articulating crane on a service vessel, shown here being used to unload equipment, is used extensively in mussel farming operations

been used for offshore macroalgae growout [21]. This device could potentially be used for mussel cultivation; however, there may be scaling issues in reaching the desired biomass.

## Mussel Species in Open Ocean Cultivation

There are several species of mussels that are cultivated in open ocean waters; however, regardless of species or location, production is currently minor by comparison with well-established nearshore production sites. In North America, small quantities of blue mussels (*Mytilus edulis)* are produced in offshore farms in New England (USA) and Atlantic Canada and Mediterranean mussels (*M. galloprovincialis*) are being grown at an offshore farm off the southern California (USA) coast [22]. In Europe, *M. galloprovincialis* are grown on submerged longlines at exposed locations in the Mediterranean coast of France [23] and in the Turkish Black Sea. Culture trials have been initiated for *M. edulis* in the North Sea off the coast of Germany, [19] and in the Belgian North Sea [24]. Other European

countries, including Portugal, Spain, Italy, and Ireland are developing strategies for offshore mussel production.

In New Zealand, where the nearshore greenshell mussel (*Perna canaliculus*) industry is well developed and highly mechanized, there is a great deal of interest in developing large-scale ocean farms, as lease sites in sheltered nearshore waters have become difficult to obtain [25]. Initial efforts at open ocean mussel farming involved moving the double longline surface technology into more exposed sites and some success was achieved in wave conditions up to 2.5 m [26]. However, failure of surface longline systems in higher energy sites has led to the development of submerged technologies and a small number of open ocean mussel farms are operating in New Zealand offshore waters, with many new farms proposed [27]. This scale of expansion is projected to provide a threefold increase in production and export earnings by 2020 [28].

While data is limited to a few locations in North America and France, there are indications that production cycles and product quality for mussels grown in open ocean waters are highly favorable. Open ocean farms off the New Hampshire coast in the northeast USA have consistently produced market-sized (55 mm) blue mussels in 12–14 months from spat settlement with meat yields ranging from 42% to 58% [9]. Similar data has been reported for blue mussels at sites off the coast of Martha's Vineyard [29]. By comparison, rope-grown blue mussels from nearby estuaries and bays can take up to 18 months to reach market size [30]. Mediterranean mussels produced at an open ocean site in California have also demonstrated excellent growth and quality, reaching market size in 6–8 months and nearly 50% meat yield [22]. Trials in the North Sea have shown that the growth conditions in the German Bight are very favorable for mussel cultivation. Market-size (50–55 mm) can be reached by 12–15 months and infestation by parasites is much lower than in nearshore sites [10]. Faster growth at offshore sites may to be due to a more stable temperature and salinity conditions and therefore lower stress, reduced turbidity, and better water exchange [20].

## Open Ocean Mussel Farming in Multiuse Facilities

Open ocean mussel farming can be practiced in isolation of other activities; however, there may be economic or environmental advantages to combining mussel culture with offshore fish farming or energy production. At a nearshore marine farming site in New Brunswick, Canada, Lander et al. [31] demonstrated better growth rates for raft-cultured mussels 100 m down current of a salmon farm than at reference sites, and was able to document that organic wastes, primarily fine particulates from feed emanating from the salmon farm contributed to the diet of the mussels. In open ocean sites, creating mussel culture "zones" in proximity to finfish farms may offset the effects of organic loading to the environment [32].

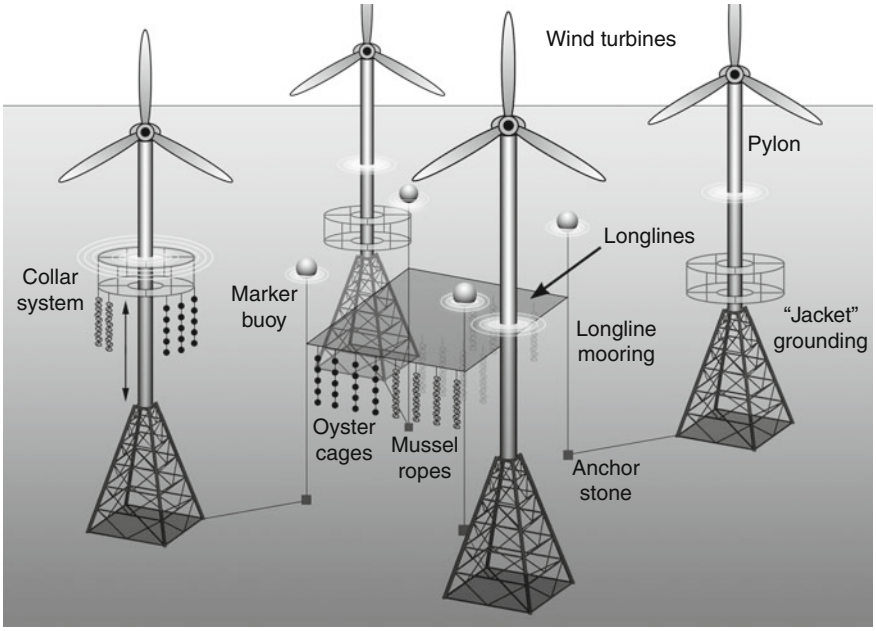
Energy installations may also provide structure for deployment of mussel culture systems. Mussels (*M. galloprovincialis*) have been harvested from oil platforms in California, USA for many years [33], and there is interest in using decommissioned offshore oil platforms as attachment points for mussel culture infrastructure.

Buck et al. [34] investigated the possibility of integrating suspension culture of oysters and mussels at existing offshore wind energy platforms in the North Sea (Fig. 5).

There are a number of advantages for conducting mussel cultivation activities within the footprint offshore wind farms. The placement of aquaculture production facilities in defined corridors between wind farm turbines eliminates the need for a separately permitted facility and reduces the space required if the two facilities were located separately [34]. Also, infrastructure for regular servicing may be shared. As both industries need a multifunctional service vessel, preferably with lifting capacities to install and change plant components and execute farming operations, and sufficient deck space to carry equipment and stock, the opportunity to share high-priced infrastructure exists [35]. Further, a combined environmental impact assessment for both users may reduce costs.

## Environmental Considerations for Open Ocean Mussel Farming

Like all forms of food production, the culture of marine species, whether practiced in land-based, nearshore, or open ocean locations will have some effect on the environment. The effect can be both negative and positive and can vary depending upon the species,




**Mussel Culture, Open Ocean Innovations. Figure 5**
A schematic of shellfish growing systems associated with wind turbine towers (from Buck et al. [34])

location, and farming practices. In the past 3 decades of marine farming in sheltered marine waters, adverse impacts from aquaculture of both molluskan shellfish and finfish have been documented, though most of the concerns and controversy are centered on finfish. Mollusk culture is generally perceived as environmentally benign or even beneficial [5]; however, there have been documented environmental impacts from nearshore mussel farming that merit consideration for development of the offshore sector.

Though mussels feed on naturally occurring seston and no external feed is provided to the organisms, deposition of feces and pseudofeces can enrich bottom sediments beneath culture systems and impact benthic communities [36, 37]. Occurrences of sediment impacts have been associated with very dense culture in shallow embayments; therefore, if offshore farms are sited in locations with sufficient depth and adequate water circulation to disperse wastes, enrichment of bottom sediments should not be an issue [7]. High-density mussel culture can also deplete the water column of planktonic food, affecting both the growth and fitness of the cultured organisms as well as naturally occurring filter feeders in the system [38]. This too, is an impact that has been observed in sheltered embayments with limited circulation and is unlikely to be an environmental issue in open ocean waters [8]. However, in very large, high-density offshore farms, depletion of food within the farm and reduced growth and condition of the stock may be an issue for producers.

Hydrodynamic alteration is another environmental effect that has been documented in sheltered embayments with high-density shellfish culture [39] and has recently been an issue of concern in New Zealand where large-scale open ocean mussel farming is in development. Plew et al. [28] reported significant current and wave attenuation and strong water column stratification at a large (230 longline) mussel farm in Golden Bay, New Zealand. The farm was located in relatively shallow water (10–12 m) and the culture organisms were suspended from the surface to a depth of 8 m, therefore, occupying nearly the entire water column. As it is likely that open ocean development will use submerged culture in much deeper water (30–100 m) with ample space above and below the culture arrays, the severity of flow modifications as observed in this study are improbable.

**Mussel Culture, Open Ocean Innovations. Figure 6**
Seed collecting rope (*black*) is attached to the backbone of a submerged longline

A legitimate environmental concern for open ocean mussel culture is entanglement of whales and other marine life in seed collection lines [40]. These collectors are either discrete lengths of line or one continuous length of rope suspended from the backbone to provide substrate for settlement of mussel larvae (Fig. 6). As this sector develops, it is important to avoid deployment of seed collection lines in the migratory pathways of endangered marine mammals or to use weak links and electronic alert systems in the farming infrastructure [41].

## Future Directions

Developments over the past 2 decades indicate that aquaculture production of mussels in open ocean environments is feasible and that opportunities exist for large-scale production [9, 10]. Conflicts with other uses can be significantly reduced, though they are not totally eliminated [34]. There is also evidence to support the premise that environmental impacts can be reduced by farming in open ocean environments [8, 36]. There is also strong indication that if sites are chosen properly, faster growth and excellent product quality can be achieved [9].

Though some technical challenges remain such as the development of large, purpose built, and highly seaworthy service vessels, obstacles to development of open ocean mussel farming are primarily economic, social, and political in nature. The scale of investment needed to establish and operate large-scale open ocean mussel farms is not well known, though it is assumed that production costs will be higher than for nearshore farming. The additional costs could be partially offset if ocean grown mussels, due to superior quality and greater consumer confidence in product safety can command a higher price [9], however, market prices are subjected to many economic externalities that are difficult to forecast. Space conflicts with the fishing industry may be an issue in some locations, therefore, involvement of local capture fishermen in industry development may be needed to gain acceptance of an alternative use of ocean space. As many countries move toward spatial planning of their territorial ocean waters, it is important to include a future vision of the potential for open ocean mussel farming in the planning process and give due consideration to compatibilities and possible synergies with other uses. Many countries also currently lack the regulatory framework for permitting open ocean farming sites. Until economic and regulatory uncertainties are resolved, entrepreneurs will be reluctant to make the level of investment needed to move this sector forward.

Ideally, development of open ocean farming should take place within the context of overall ocean management and marine spatial planning in order to assure compatibility with other uses and consistency with broader goals to restore and sustain the health, productivity, and biological diversity of the oceans.

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