

Review

Plasticity and Multiplicity of Trophic Modes in the Dinoflagellate *Karlodinium* and Their Pertinence to Population Maintenance and Bloom Dynamics

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Abstract: As the number of mixotrophic protists has been increasingly documented, “mixoplankton”, a third category separated from the traditional categorization of plankton into “phytoplankton” and “zooplankton”, has become a new paradigm and research hotspot in aquatic plankton ecology. While species of dinoflagellates are a dominant group among all recorded members of mixoplankton, the trophic modes of *Karlodinium*, a genus constituted of cosmopolitan toxic species, were reviewed due to their representative features as mixoplankton and harmful algal blooms (HABs)-causing dinoflagellates. Among at least 15 reported species in the genus, three have been intensively studied for their trophic modes, and all found to be phagotrophic. Their phagotrophy exhibits multiple characteristics: (1) omnivory, i.e., they can ingest a variety of preys in many forms; (2) flexibility in phagotrophic mechanisms, i.e., they can ingest small preys by direct engulfment and much bigger preys by myzocytosis using a peduncle; (3) cannibalism, i.e., species including at least *K. veneficum* can ingest the dead cells of their own species. However, for some recently described and barely studied species, their trophic modes still need to be investigated further regarding all of the above-mentioned aspects. Mixotrophy of *Karlodinium* plays a significant role in the population dynamics and the formation of HABs in many ways, which thus deserves further investigation in the aspects of physiological ecology, environmental triggers (e.g., levels of inorganic nutrients and/or presence of preys), energetics, molecular (genes and gene expression regulations) and biochemical (e.g., relevant enzymes and signal molecules) bases, origins, and evaluation of the advantages of being a phagotroph.

Keywords: *Karlodinium*; trophic modes; phagotrophy; mixotrophy



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1. Introduction

Microalgae are an important group in terms of global primary productivity. Those microalgae that spend their time on vegetative growth in the water column are categorized as phytoplankton, a counterpart of zooplankton in aquatic ecology [1]. As the terms imply, autotrophy or phototrophy is the most important trophic mode in microalgae or phytoplankton and thus the focus of research on microalgae [1–3], which is reasonable and fair in terms of their major function in aquatic ecosystems as primary producers. However, other trophic modes have been found in many groups or species of microalgae and have attracted increasingly more attention from the scientific community during the last several decades because these non-autotrophic modes have been, or will be, proven to be vital strategies for the population survival and development (e.g., blooms) of phytoplankton [4].

Among 2400 valid species of dinoflagellates, about 50% are strictly heterotrophic, while the other half of species obtained and maintained the ability of photosynthesis [5].

Independent of the number of species, these photosynthetic dinoflagellates occupy an essential place in primary production, particularly in coastal and estuarine ecosystems [6]. As the dark facet of primary producers, dinoflagellates are also the crucial perpetrators of harmful algal blooms (HABs) forming species, given that they are responsible for 75% of documented HABs [7]. However, intriguingly, photosynthetic dinoflagellates are generally of relatively lower photosynthetic capacity per unit of biomass and exhibit lower growth rates in comparison to many of their competitors, such as diatoms [8,9]. Thus, dinoflagellates must have other strategies to balance this competitive disadvantage. Mixotrophy, a nutritional strategy by which organisms are able to obtain nutrients and/or energy by both phototrophic autotrophy and heterotrophy [10–12], is one of these strategies that enhance growth rates via obtaining energy from either dissolved organic compounds [13] or particulate preys [14]. The mixotrophic protists that play roles of both primary producer and consumer have been widely investigated from different aspects [11,12].

The genus *Karlodinium* J. Larsen was erected from the genus *Gymnodinium* in 2000 because of the characteristics of their apical groove, ultrastructure, and partial large subunit rDNA sequences [15]. Species of *Karlodinium* are well known for forming HABs and thus causing the consequent fish-killing events [16–18]. The genus includes at least 15 species to date (Table 1). The distribution of the genus *Karlodinium* spreads over four oceans [16] (also see Ocean Biogeographic Information System, <https://obis.org/taxon/231789>). *Karlodinium veneficum* (original name: *Gymnodinium veneficum*; synonym: *Karlodinium micrum*, *Gymnodinium micrum*, *Gyrodinium galatheanum*, *Woloszynskia micra*, and *Gyrodinium estuariale*) is the type species and also the most intensively and extensively investigated one in the field and the laboratory [19–23]. Species in the genus of *Karlodinium*, such as *K. veneficum*, *K. armiger*, *K. corsicum* and *K. aculat*, have been reported to be associated with many toxic events and caused mortality of fishes, mussels and zooplanktons [18,24–30]. Multiple types of toxins have also been detected from these species. The toxins produced by *K. veneficum* are termed as karlotoxins [25] and at least 12 natural analogs of karlotoxins have been identified to date [25,31–33]. *Karlodinium conicum* was also proved to produce karlotoxin [34,35]. From *K. armiger*, however, a different species of toxin, karmitoxin, has been chemically characterized [36,37]. Besides, the presence of some types of NSP toxins in *K. corsicum* has also been testified by mouse tests [27].

Table 1. A collection of *Karlodinium* species regarding their bloom threat, toxicity, trophic modes, and associated mechanisms.

Species	Former names and/or taxonomic synonyms	Distribution	Blooms	Toxicity	Autotrophy	Osmotrophy	Phagotrophy	Peduncle-like structure
<i>K. armiger</i>	\	Alfacs Bay, Ebro Delta, NW Mediterranean [38]	Yes [29,39]	Yes (Karmitoxin) [37]	Yes	?	Yes [40,41]	Have a peduncle [38]
<i>K. australe</i>	\	North-eastern Tasmania, Port Phillip Bay (Victoria), South Australia and Tuggerah Lakes [28] and Singapore [42,43]	Yes [18]	Yes [18,28]	Yes [28]	?	Yes [28]	Have a thick, tubular peduncle-like structure [28]
<i>K. antarcticum</i>	\	Southern Ocean [43]	?	No [34]	Yes [43]	?	?	Have a tube-shaped structure [43]
<i>K. azanzae</i>	\	Manila Bay, Philippines [44]	?	Yes [44]	Yes [44]	?	Yes [44]	Have a peduncle [44]
<i>K. ballantinum</i>	\	Mercury Passage, Tasmania, Australia, and Tyrrhenian coastal waters [43] and the Mexican Pacific [45]	?	?	Yes	?	?	Have a tube-shaped structure [43]
<i>K. conicum</i>	\	Southern Ocean [43]	?	Yes (KmTx) [34]	Yes	?	?	Have a tube-shaped structure [43]
<i>K. corrugatum</i>	\	Southern Ocean [43]	?	No [34]	Yes	?	?	?

Table 1. Cont.

Species	Former names and/or taxonomic synonyms	Distribution	Blooms	Toxicity	Autotrophy	Osmotrophy	Phagotrophy	Peduncle-like structure
<i>K. corsicum</i>	<i>Gyrodinium corsicum</i>	Corsica (France), Tyrrhenian Sea and the Spanish Alfacs Bay of Mediterranean Sea [29,46]	Yes [24]	Yes [26]	Yes	?	?	Have a ventral plate [47]
<i>K. decipiens</i>	<i>Karenia digitate</i>	from coastal Tasmania southward to the north polar front, and western European Atlantic waters (Bilbao, Spain) [43]	?	No [34]	Yes	?	?	Have a tube-shaped structure [43]
<i>K. digitatum</i> ¹	<i>Karenia digitata</i>	Japan coastal waters of Hong Kong, Fujian and Guangdong's Southern, China [48,49]	Yes [49]	Yes [49]	Yes	?	?	Have small finger-like extensions [49]
<i>K. elegans</i>	\	Pingtang coastal water, East China Sea [50]	?	No [50]	Yes [50]	?	?	Have a tube-like structure [50]
<i>K. gentienii</i>	\	The Atlantic coast of Brittany [51]	Yes [51]	Yes [51]	Yes	?	?	Have a tube-shaped structure [51]

Table 1. Cont.

Species	Former names and/or taxonomic synonyms	Distribution	Blooms	Toxicity	Autotrophy	Osmotrophy	Phagotrophy	Peduncle-like structure
<i>K. veneficum</i>	<i>Gymnodinium veneficum</i> ; <i>Karlodinium micrum</i> ; <i>Gymnodinium micrum</i> ; <i>Gyrodinium galatheanum</i> ; <i>Woloszynskia micra</i> ; <i>Gyrodinium estuariale</i> [38,52]	Cosmopolitan [16]	Yes [29,39,53]	Yes [54]	Yes [20]	Yes	Yes [16,20,21]	Have a peduncle [38]
<i>K. vitiligo</i>	<i>Gymnodinium vitiligo</i> ? <i>K. veneficum</i> [38]	?	?	?	Yes	?	?	?
<i>K. zhouanum</i>	<i>K. jejuense</i>	Widely spread over the coastal waters of China [55,56]	Possible [57]	?	Yes [56]	?	?	Tube-like structure in intercingular region [56]

Note: “\” indicates none. “?” indicates that there is no explicit record in the literature.

The trophic modes of *Karlodinium* have been studied for several decades, and were found to be diverse and typical. However, new findings have been made recently and improved our understanding of this genus, which comprises a group of mixoplankton. At least four *Karlodinium* species have been confirmed to be mixotrophic, namely *K. veneficum* [20,58], *K. armiger* [40,41], *K. aculat* [28] and *K. azanzae* [44], by now. Among these proven mixotrophic species, some exhibit plastic and multiple trophic modes, as well as wide spectrum of prey size and varieties, i.e., *K. armiger* and *K. veneficum* [16,59]. Other species, while direct evidence about their mixotrophy is lacking at present, were also reported to possess peduncle-like structures (Table 1), namely the instrument for phagotrophy, such as *K. gentienii* [51] and *K. zhouanum* [56].

Because possibly all *Karlodinium* species have potential for mixotrophy and the highly flexible trophic modes may be a vital trait of this and other similar groups of dinoflagellates in their ecology and evolution, here we review the knowledge advancement in understanding the trophic modes of dinoflagellates in general and *Karlodinium* in particular, with the hope of inspiring further investigations on the genetic, cellular, physio-chemical, and ecological mechanisms of mixotrophy in dinoflagellates (HABs-forming groups particularly) by putting forward our insights and suggestions about the interesting topic.

2. Trophic Modes of Dinoflagellates

In addition to autotrophy or phototrophy, many free-living dinoflagellates live as either heterotrophs or mixotrophs [5,60]. Mixotrophic modes can be further categorized as amphitrophic (heterotrophy or autotrophy alone is sufficient for nutrition) and mixotrophic *sensu stricto* (both forms of nutrition are required) [61].

Dinoflagellates have evolved multiple heterotrophic nutritional strategies [61]: (1) osmotrophy (or resorption), by which the organic macronutrients are taken up by direct passage through the plasma membrane, (2) saprotrophy, a chemoheterotrophic process of digesting organic matter extracellularly and (3) endocytosis, which includes pinocytosis (cell drink, a mode of endocytosis by which liquid organic matter is taken up into the cell by invaginating of the cell membrane, and forming a small vesicle inside the cell) and phagocytosis or phagotrophy (cell eating, the endocytosis of particulate food).

Generally, there are three types of feeding mechanisms of phagotrophy that dinoflagellates use to uptake food particles (including intact cells): (1) direct engulfment (phagotrophy *sensu stricto*), i.e., a cell phagocytizes an entire food particle, including the prey cell membrane [61,62], (2) tube feeding, i.e., the feeding cells use an feeding appendage to suck food particles (e.g., *Peridiniopsis berolinensis*) [63,64], and (3) pallium feeding, i.e., some species use a feeding veil, namely pallium, to surround and digest the prey outside the cell body of the predator, then the liquefied cytoplasmic content of prey is taken up by the predator, leaving only an empty wall or frustule (e.g., *Zygabikodinium lenticulatum*, *Oblea rotunda* and *Protoperidinium conicum* [64–66]).

Direct engulfment, or phagocytosis *sensu stricto*, of dinoflagellates seems to be mainly found in athecate dinoflagellates, like *Blastodinales*, *Gymnodinales*, *Noctilucales*, and *Oxyrrhinales* [61,67]. However, it remains unclear whether this apparent “preference” is due to the higher flexibility or elasticity of the cells of naked species than armored species. In addition, special feeding organelles, such as tentacles, lobopodia and peduncles, were usually found in the sulcal region near the flagellar groove [61].

Tube feeding has been observed to use two types of feeding tubes in dinoflagellates: the peduncle (a protoplasmic strand protruding from the mid-ventral area of the sulcus to connect predator and prey, e.g., *Paulsenella*) [61,68,69], and the phagopod (a non-cytoplasmic feeding tube, e.g., *Amphidinium cryophilum*) [70]. Myzocytosis is a kind of tube feeding by which the feeding cells suck out the contents of prey cells by leaving the plasma membrane outside the predator. The prey plasmalemma is not taken up, and thus the prey cytoplasm is bounded only by the vacuolar membrane in the food vacuole. This mode of nutrition was first described in the naked dinoflagellate *Gyrodinium vorax* Biecheler [62]. The terminology for the uptake organelle of myzocytosis has not been uniformed, and it

has been variously referred to as feeding tubes or peduncles [61], and in this review, we use the term “peduncle” to refer to the uptake organelle of myzocytosis. The peduncle was reported to be formed by the emergence of a preformed “microtubular basket” which consists of plates of microtubules [69,71]. Based on the light and electron microscopic observations on *Pausenella* sp., Schnepf et al. (1985) suggested that the food uptake was driven by a hydrostatic gradient which might be attributed to rhythmical ion pumping and based on the existence of a common cavity and the sphincters [71]. In contrast to most suctorian tentacles, peduncles are generally not permanently protruded [61,69], and are usually invisible in predators not feeding on food [69]. The length of feeding tubes also differs in species and even varies in a single cell with feeding status [61]. No prey size spectra are confirmed for tube feeders in the literature, but some researchers have pointed out that the prey size seems not to have an upper limit, as studies reported the ingestion ability of *K. veneficum* and *K. aculat* on rotifer, copepod eggs, and even tissues of fish [16,72,73]. The strictly heterotrophic species *Pfiesteria shumwayae* was found to exhibit lethal effect on fish by myzocytosis, also named “micropredation” [74], a trophic strategy in which a predator feeds on a rather large prey and one feeding individual attacks more than one prey during its life span and attacks the prey intermittently without necessarily eliminating its fitness (e.g., mosquito) [75]. However, both direct engulfers and pallium feeders have prey size spectra restricted to the volume capacity of the predator cell [64].

The feeding processes of phagocytosis were described by several steps including pre-capture behavior, capture, and prey manipulation [64]. While in pre-capturing, dinoflagellates swimming faster than their prey are referred as the “searching type” and those being able to catch the faster-moving prey are described as the “trapping type” [64]. Search type is induced by chemical substances released from the injured prey and is independent of prey size [63,76]. It is demonstrated that dinoflagellates of similar size but with different speed in comparison to preys, swimming characteristics, and feeding strategies (peduncle vs. tow line) have substantially different responses to the introduction of preys [77]. The feeding dinoflagellates usually capture preys using some specialized appendages named “capture filament” or “tow filament”. The capture filament of *Peridiniopsis berolinensis* is a thin filament that originates from the ventral region of the cell near the sulcus [63]. Once the filament anchors to the prey, it retracts, brings the prey closer to the predator (e.g., *Protoperidinium* and *Diplosalis* group) or contracts entirely, and thereby drags the prey to the sulcal region of the predator (e.g., *Gyrodinium*) [78]. After capturing the prey, most dinoflagellates consume the prey immediately, but the manipulation of prey may differ with other feeding mechanisms [14,78].

Certain dinoflagellates may utilize kleptochloroplasts (transiently alien chloroplasts) obtained from preys [79]; this nutrient strategy is termed kleptochloroplastidy [80]. Myzocytosis is the proven method to acquire kleptochloroplasts from preys [61,81]. Hansen (1998) reviewed a few species that lack chloroplasts but are capable of sequestering chloroplasts from other phytoplankters and then using the “stolen” chloroplasts for photosynthesis [82]. This kind of mixotrophy has been reported among some species belonging to the naked genera *Amphidinium* and *Gymnodinium* [82–84]. It is noteworthy that the latter may contain species from *Karlodinium*, as this genus had not been separated from *Gymnodinium* until 2000 [15]. Li et al. found fragmental pigments from cryptophycean prey in *K. veneficum* that had been ingested with the prey for 41 h, suggesting some chloroplasts of prey could be retained by the dinoflagellate [20].

3. Autotrophy of *Karlodinium*

All *Karlodinium* species have the ability of photosynthesis (Table 1). *Karlodinium veneficum* and *K. armiger* are the best-studied species that have haptophyte origin chloroplasts [20,21,40,59,85,86]. Phototrophic growth rates of *K. armiger* are quite low (a maximum of 0.01 and 0.10 d⁻¹), even at high irradiances [40,59]. In comparison, *K. veneficum* grows faster than *K. armiger* photosynthetically without prey, with growth rates ranging from 0.17 to 0.36 d⁻¹ [87]. In some cases, the growth rate of *K. veneficum* may even elevate up to

0.55 d^{-1} in the light without prey [85]. The photosynthetic growth rate of *K. veneficum* is significantly affected by temperature and salinity [23]. At least some strains of *K. veneficum* were better adapted to “low-light” conditions than were *K. armiger*, whereas characteristics of *K. armiger* were more suitable to cope with “high-light” [54].

Karlodinium aculat grows poorly in the normal conditions without providing food. The monoculture of *K. australe* grown in laboratory and Gse medium stabilized at low concentrations (10^2 – 10^3 cells mL^{-1}) and failed to reach higher cell concentrations [28]. Lim et al. obtained similar results from a *K. austral* bloom in the cage-farming region of the West Johor Strait of Malaysia (0.31 – 2.34×10^3 cells mL^{-1}). However, *K. veneficum* could reach extremely high cell densities (2 – 3×10^5 cells mL^{-1}) in laboratory cultures [88]. These observations suggest that different species of *Karlodinium* may also differ in their phototrophic growth potential. In contrast to the relatively poor autotrophic ability, the genus is successful in forming harmful blooms. Thus, other nutritional strategies may play a key role in population competition and deserve further investigation.

4. Osmotrophy of *Karlodinium*

Osmotrophy, i.e., the uptake of dissolved organic compounds, has been shown to be an efficient nutritional strategy for algae. *Karlodinium veneficum* is the most studied species in *Karlodinium* on osmotrophy. Cell-surface proteolytic activity (leucine aminopeptidase) was detected in *K. veneficum* and suggested to play a role in obtaining nutrition by obtaining amino acids for assimilation, while, alternatively, released amino acids may be degraded by cell-surface amino acid oxidases to provide ammonium, which can be assimilated as a source of nitrogen [89]. Solomon and Glibert found that urease activity in *K. veneficum* was significantly higher than that in other species (including *Heterocapsa triquetra*, the cryptophyte *Storeatula major*, and the haptophyte *Isochrysis* sp) on both a per cell basis and a per cell volume basis [90]. Harmful dinoflagellates like *K. veneficum* may be better suited to utilize urea than other species do according to their high urease activity and large intracellular urea pools, which may explain why these harmful dinoflagellates proliferate rapidly in the water bodies with plenty of urea [90].

Osmotrophy may be an important and ubiquitous trophic strategy for all species in *Karlodinium*, because almost all phytoplankton are osmotrophs in some parts, not least by virtue of being auxotrophic; many need external sources, e.g., vitamins [91]. Phytoplankton exhibit non-auxotrophic osmotrophy to a significant level, mostly in relation to the uptake of primary metabolite compounds, especially amino acids [54]. It has been reported that many dissolved organic compounds, such as amino acids (e.g., glutamine, leucine, thymidine, aspartic acid), carbohydrates (e.g., glucose) and other organic compounds (e.g., acetic acid, coumaric acid, glycerol), can be used as carbon and nitrogen sources, which are commonly released by the algae themselves or bacteria [92–96].

5. Phagotrophy of *Karlodinium*

5.1. *Karlodinium veneficum*

Karlodinium veneficum exhibited increased ingestion rate on eubacteria when phosphate was limited, which may be an important nutrient-acquiring strategy when inorganic nutrient is limited [97]. *Karlodinium veneficum* was also reported to ingest various kind of small algae by phagocytosis, including *Chroomonas salina*, *Cryptomonas appendiculata*, *C. calceiformis*, *C. maculata*, *Hemiselmis brunnescens*, *H. rufescens*, *Hemiselmis* sp., *Rhinomonas reticulata*, *Rhodomonas salina*, *Rhodomonas* sp., *Storeatula major*, and *Isochrysis galbana*, and most of them are cryptophytes [20–22,85,86,98,99]. The direct engulfment of whole cells of *Storeatula major*, a species of cryptophyte, by *K. veneficum* and the associated feeding processes were initially documented via video recording under light microscope [20]. This phagocytosis process was described to have three typical steps [20]: (1) Pre-capture behavior. After adding cryptophyte as prey, most *K. veneficum* cells increased the swimming speed, and some began to swim around the prey; (2) Capture. Generally, *K. veneficum* cells formed a protrusion, which was near the flagellar pores in the sulcal region, and attached to the prey. Once the protrusion contacted the prey cell, phagocytosis began.

In some cases, a thin capture filament projected from the extending sulcal region in the epicone was observed, and then the filament captured and drew the prey cell to the surface of the dinoflagellate in the sulcal region (Figure 1a, SEM micrographs were adopted from Place et al. (2012) [72]; (3) Prey manipulation. After capturing prey firmly, *K. veneficum* usually stopped swimming to draw the whole cell of prey into the dinoflagellate cell through the protrusion. During the process of feeding, a “feeding gap” appeared to form along the cingulum near the flagellar pores and a pair of “lip-like” protrusions (i.e., peduncle) was observed (Figure 1b) [20]. The engulfment behavior usually took 2 to 3 min at room temperature (20 °C) and, when the ingestion was completed, *K. veneficum* cells resumed swimming and were able to find and phagocytize another prey cell.

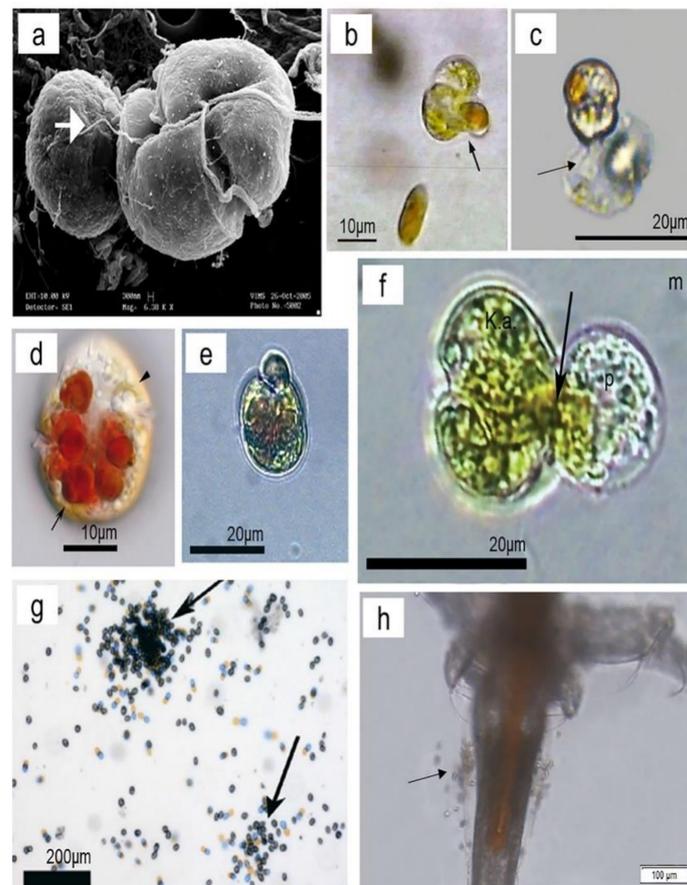


Figure 1. The phagotrophic behavior of *Karlodinium*. (a) SEM of *K. veneficum* feeding on *Rhodomonas* sp. The thin filament is marked with an arrow (the photo was modified from Place et al. [72]). (b) A cell of *K. veneficum* was engulfing whole cells of *Storeatula major*. The “lip-like” protrusion was observed to gradually move laterally along the prey surface, which causes further engulfment of the prey cell (the photo was modified from Li et al. [20]). (c) *Karlodinium veneficum* ingest a dead cell of con-species by myzocytosis using peduncles. *Karlodinium veneficum* was searching for cytoplasm by opening the peduncle (arrow) widely in the dead cell (the photo was modified from Yang et al. [16]). (d) Subsurface ventral view of *K. australe* after feeding overnight on *Rhodomonas salina*. Note light yellow-green chloroplasts (arrowhead) and red food vacuoles (arrow, the photo was modified from de Salas et al. [28]). (e) *Karlodinium armiger* was ingesting the cryptophyte *Rhodomonas salina* by direct engulfment (the photo was modified from Berge et al. [59]). (f) *Karlodinium armiger* was ingesting the raphidophyte *Fibrocapsa japonica* (p) by myzocytosis (the photo was modified from Berge et al. [59]). The peduncle was shown by the arrow. (g) Aggregations (arrows) of *K. armiger* cells in cultures fed thecate dinoflagellate *Prorocentrum minimum* (the photo was modified from Berge et al. [59]). (h) Aggregations (arrows) of *K. veneficum* cells fed an injured brine shrimp *Artemia salina* (the photo was modified from Yang et al. [16]).

Sheng et al. also represented the phagocytosis process of *K. veneficum* on *Storeatula major* and paid more attention to its pre-capture behavior. When presented with *S. major*, the velocity, radius, and pitch of *K. veneficum* reduced, but its angular velocity increased [58,77]. The feeding cells of *K. veneficum* significantly reduced their usual vertical migration, probably to remain in the vicinity of their preys [58,100].

A peduncular microtubular strand was observed in *K. veneficum* cells and believed to be a tube feeder; however, the small sized preys such as eubacteria and cryptophytes were obviously ingested by direct engulfment [20,58], indicating an alternative function of the microtubular strand. Although *K. veneficum* was also observed to have the potential to feed on the diatom *Melosira* and copepod *Acartia tonsa* [72], direct evidence of feeding behavior using peduncles has not been captured. Recently, we observed *K. veneficum* ingested preys via myzocytosis using the peduncle [16], in which the entire feeding process was much the same as that observed in direct engulfment except that only the cytoplasm of prey (cells or larger multicellular individuals) was sucked into *K. veneficum* cells through the peduncle in myzocytosis (Figure 1c) and the time expenditure of myzocytosis, which varied from several seconds to a few minutes, was relatively shorter than that of the direct engulfment on small-sized prey [16]. As the ingestion proceeded, the cell volume of *K. veneficum* gradually increased [16]. Profiting by this mechanism, the prey size spectra would have no upper limit. We demonstrated that *K. veneficum* is virtually an omnivorous feeder, as it could feed on both live and dead bodies/cells of phytoplankton (the dinoflagellates *Margalefidinium polykrikoides*, *Akashiwo sanguinea*, and *Alexandrium leei*, the cryptophyte *Rhodomonas salina*, and the haptophyte *Isochrysis galbana*) and animals (the finfish *Oryzias melastigma*, brine shrimp *Artemia salina*, and rotifer *Brachionus plicatilis*). Importantly, *K. veneficum* also exhibited cannibalism (i.e., feeding on dead cells of its own species), which implies that the dead and weak cells of *K. veneficum* can be ingested by the live cells to recycle nutrients contained in the eaten cells [16]. Cannibalism is one of the simplest trophic interactions [101]. The advantageous aspect of this particular type of phagotrophy is that it allows an efficient nutrient transfer because of the well-matched nutritional value between the food and consumer [102]. We also observed that *K. veneficum* could survive at a lower cell density, without inorganic nutrients supplementing the culture medium, for a year, which was obviously attributable to the cannibalistic phagotrophy [16]. Cannibalism was also observed in *Protoberidinium* when cell abundances were high, and in *Oxyrrhis* when “victim” and “cannibal” differed in sufficient cell size-classes [66,102]. These observations suggest that cannibalism may be a mechanism of withstanding prolonged starvation.

The ingesting ability of *K. veneficum* is affected by environmental factors such as irradiance. It was observed that *K. veneficum* did not exhibit phagocytosis without light and the ingestion rate increased drastically when irradiance rose up to $\sim 50 \mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ [20].

5.2. *Karlodinium australe*

Karlodinium australe has also been known to phagocytize particulate foods in food vacuoles since the species was initially described [28]. A thick and tubular peduncle-like structure of this organism was observed in the sulcal region [28]. Phagotrophy in *K. australe* was captured when autotrophically grown cultures were provided with live *R. salina* cells as food (Figure 1d) [28]. However, the intact feeding behavior has not been captured. A recent study further investigated the feeding mechanism and ecological implication of the phagotrophic mixotrophy of *K. australe*. *Karlodinium australe* is a phagotroph that can ingest preys via direct engulfment or tube feeding. In accord with its flexible phagotrophic modes, *K. australe* is also an omnivorous mixotroph. Except *R. salina*, a diverse range of organisms could be ingested, such as microalgae (*Isochrysis galbana*, *Margalefidinium polykrikoides*, *Karenia mikimotoi* and *Gymnodinium catenatum*) and zooplankton (*Artemia salina* and *Brachionus plicatilis*) [73].

5.3. *Karlodinium armiger*

Karlodinium armiger is an omnivorous and obligate mixotroph. It seems that *K. armiger* obtain essential growth factor or substance through phagotrophy [59]. This species can ingest many types of preys (except for almost all Bacillariophyceae tested), but yield the highest growth rates when offered cryptophytes as prey [40,59]. However, *K. armiger* cannot grow and survive by feeding in complete darkness or at dim light, even by feeding adequate amounts of preys regularly [59], indicating that *K. armiger* cannot grow on as complete phagotrophy.

Under light microscope, the feeding mechanism of *K. armiger* was occasionally assumed to be direct engulfment (i.e., phagocytosis *sensu stricto*) while it was feeding on small-sized cells of prey; however, it would use tube feeding (myzocytosis) when the preys were larger or thecate [59]. Rigid cell coverings seem to set a barrier to grazing; thus, species like diatoms and thecate dinoflagellates may not be appropriate food [59].

Some details of the phagotrophy process in *K. armiger* were also documented by Berge et al. [59]: *K. armiger* also displayed distinct and intense pre-capture behavior by increasing swimming speed and frequently changing swimming direction before ingestion. This pre-capture swimming behavior has also been documented in other phagotrophic dinoflagellates [63,76,103,104]. The predator cell usually encountered a prey cell with its apical part. After contacting with a prey cell, the predator slowed down the swimming speed. During this stage, less than half of the prey cells (*R. salina*) were captured. Occasionally, a capture filament, an up to 10 μm long structure, which has also been reported in other phagocytosing dinoflagellates, such as *Peridiniopsis berolinensis* [63] and *K. veneficum* [19,20], was observed to attach the prey. When the capture succeeding, the predator placed its sulcal area, where the phagocytosis took place, facing the prey and revolved around its anterior–posterior axis. During this feeding stage, a small protrusion sometimes appeared. However, most preys and predators often established close contact immediately without any signs of protrusion. Often, the whole *Rhodomonas* cell was apparently engulfed or sucked into a food vacuole (Figure 1e) [59]. Occasionally, the cytoplasm was separated from the periplast of cryptophyte and taken up through the sulcus, leaving the periplast behind [59].

However, it differed somewhat from the feeding sequence of ingesting intact cells of *R. salina* when the predator cells fed on relatively large preys ($> 10 \mu\text{m}$). During feeding on large preys like the raphidophyte *Fibrocapsa japonica*, only a small part was sucked into a food vacuole. The cytoplasm separated from the cell membrane of the prey and flowed into food vacuoles of the predator through a narrow part (3–4 μm thickness) of the sulcal area (Figure 1f) [11]. This behavior resembled myzocytosis or tube feeding [59].

Karlodinium armiger feeds on preys using an unnoticeable feeding tube (peduncle) which allows for ingestion of larger food particles [59]. However, Bergholtz et al. reported the presence of a peduncular microtubular strand in *K. armiger* [38]. The optimal prey size for *K. armiger* was about 13 μm , a size class which is close to the predator and contributes higher ingestion rates. Smaller preys ($< 8 \mu\text{m}$) resulted in lower ingestion rates (20–24 $\text{pg C cell}^{-1} \cdot \text{d}^{-1}$), but still contributed to fairly high growth rates (0.35–0.45 d^{-1}). Although *K. armiger* can feed on preys in a large size spectrum [40], maximum growth rates relied more on prey taxa (cryptophytes) rather than on prey size when the food was saturated [40].

Several cells of *K. armiger* often attacked and fed on prey cells simultaneously [59]. When *K. armiger* reached higher cell densities, aggregates of predator cells swarming intensely around prey cells were easily recognized (Figure 1g) [71]. Aggregates led to fairly high swimming speeds of other *K. armiger* cells in the culture as these cells were obviously attracted to the preys. Such aggregation of predator cells around prey indicated a chemical attraction. Both mobile and immobile cells were observed to be captured and ingested [59]. We also observed the same aggregation of predator cells around a prey in *K. veneficum* (Figure 1h) [16].

The feeding mechanism of *K. veneficum* and *K. armiger* indicates that mixotrophic species of *Karlodinium* may be omnivorous phagotrophs with a relatively wide range of

prey species and prey size spectrum than previously recognized. A newly identified species, *K. azanzae*, was also demonstrated to be phagotrophic and able to feed on invertebrates by micropredation [44]. However, direct evidence of myzocytosis feeding, for most other species of *Karlodinium*, has been absent. Whether the presence or absence of a trophic mode-relevant trait in one, but not in another, species of *Karlodinium* was really caused by interspecific genetic differences, or was due to imbalanced investigations, definitely deserves more intensive study.

6. Evolution of the Feeding Mechanisms in *Karlodinium*

The feeding mechanism of *Karlodinium* seems to be plastic and of more than one type (e.g., *K. veneficum* feeding by direct engulfment and myzocytosis). According to most studies, only one feeding mechanism was found in a given dinoflagellate species [64]. The flexible feeding mechanisms of *Karlodinium* may lead a new discovery and provide a novel view of the evolution of feeding mechanisms in dinoflagellates, but it is clear that this aspect cannot be adequately summarized due to the current status of knowledge.

Cannibalism of other dinoflagellates has been reported, such as *Fragilidium*, *Peridiniopsis*, *Protoperidinium*, *Pfiesteria*, and *Oxyrrhis* [63,66,102,105–107] and may be widespread in more dinoflagellates, particularly those that are strictly heterotrophic. Cannibalism has been speculated to have particular implications during the evolution of sex because self-ingestion without self-digestion may have led to the evolution of diploidy [108].

Phagotrophy, the internalization of photosynthetic organisms by a eukaryote in a general sense, is essential for the occurrence of present-day endosymbiotic algae and kleptoplastid-containing protists, and even for the origin of plastids themselves [109]. Analysis of field data revealed that up to 40–60% of plankton which have been traditionally labelled as microzooplankton (non-) are actually non-constitutive mixotrophs. They are mixotrophs lacking a constitutive ability of photosynthesis, and thus, can employ acquired chloroplastids for phototrophy other than phagocytose for nutrients [110]. It is interesting that the evolutionary histories of chrysophytes and dinoflagellates, two groups containing the largest amounts of phagotrophic species, can be traced back to the early Paleozoic [111]. This suggests that mixotrophy, or multiple trophic modes, may be a primitive state, and also be indispensable for long term evolutionary success [112]. However, this aspect largely continues to be an unexplored area.

There may be close relationships between phagotrophy and toxicity/allelopathy of *K. veneficum*. Phagotrophy could not be an isolated aspect of the physiological ecology of phytoplankton. It may have coevolved with other physiological capabilities in many taxa, such as the ability to use dissolved organic material and allelopathic tendencies [113]. Many toxic algae have been proved to be phagotrophic or closely related to the known phagotrophs. The toxicity of *K. veneficum* in different strains exhibited a decreasing order that perfectly coincided with the increasing order of laboratorial culturing time [88]. It seems that the toxicity of *K. veneficum* may have receded because of the lack of prey.

7. Mixotrophy in Regulating Population Dynamics and HABs Formation of *Karlodinium*

The significance of mixotrophy in phytoplankton has been increasingly emphasized in recent years. In 2016, a new functional grouping of planktonic protists in an ecophysiological context was proposed to recognize the value of mixotrophy in euphotic aquatic systems and to align with the traditional dichotomy of phytoplankton and zooplankton: (1) phago-heterotrophs as protists lacking photosynthetic autotrophic capacity, (2) photoautotrophs as protists lacking phagotrophic capacity, (3) constitutive mixotrophs (CMs) as phagotrophs with an inherent capacity for phototrophy, and (4) non-constitutive mixotrophs (NCMs) as phagotrophs acquiring their phototrophic capacity by ingesting specific (SNCM) or general non-specific (GNCM) preys [114]. Given that mixotrophs differ widely in their biology, it is apparent that they are also different in their ecological niche and their implications on ecosystem processes [115]. CMs, combining functions of both phagotrophy and phototrophy, are supposed to have the capability to hold the high ground in an ecosystem,

ultimately triggering a large area of blooms. Indeed, constitutive mixotrophy has been considered as a major trophic mode for harmful dinoflagellate species in eutrophic coastal waters [4]. Moreover, mixotrophic species tend to dominate in more-mature systems, such as established eutrophic systems and oligotrophic systems in temperate summer, with their flexible nutritional supplies [116]. The mixotroph-dominated ecological structure differs radically in energy flow and material cycling, which is reflected in the shortened and more efficient transformation from nutrient regeneration to primary production. In severe eutrophic water bodies, bloom-forming phytoplankton with mixotrophic mode may sometimes decrease energy flowing to higher trophic levels and thus simplify the food web [115]. Moreover, mixotrophic protists can also take advantage of bacterial production to support primary production [116]. In view of the important role of mixotrophic protists in the marine ecosystem, “mixoplankton” was proposed and emphasized as a new paradigm for marine ecology and is believed to offer a better understanding on the microbial trophic dynamics and the biological pump, along with “phytoplankton” and “zooplankton” [116,117]. This conception may become a new research hotspot.

Mixotrophy is supposed to be a major contributor to the population dynamics of the *Karlodinium* species. Dinoflagellates with different trophic modes may indicate that they employ different survival strategies and occupy different ecological niches, and the phagotrophic tendencies of *Karlodinium* may partially explain some aspects of their bloom dynamics and population ecology. On one hand, phagotrophy may play an important role for phagotrophs in maintaining their population in environments of low light intensity and low nutrient availability [118] via acquiring limiting elements from prey. On the other hand, even in eutrophic habitats, phagotrophic mixotrophs may attain growth higher than that which they could reach in a strict phototrophic mode [4]. Phagotrophy can also contribute to a better budget of essential and major nutrients (C, N and P) in these species. It was documented that the prey-ingestion of *K. armiger* helped to acquire essential inorganic nutrients to stimulate the photo-synthetic capability under nutrient limitation, as it grew very slowly in standard growth medium ($f/2$) and light without prey, but grew dramatically faster ($\mu = 0.65 \text{ d}^{-1}$) when fed preys [119]. *Karlodinium veneficum* also grew much faster with prey than it did strictly autotrophically [20,85]. Adolf et al. studied the balance of autotrophy and heterotrophy of mixotrophic growth of *K. veneficum* [85]. It turned out that the mixotrophic growth of *K. veneficum* was dominated by heterotrophic metabolism, and photosynthesis continued at a lower rate, suggesting a shift toward heterotrophy during grazing. It is confirmed that photosynthesis contributed 27–69% of the gross C uptake with an irradiance at $200 \mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ and a daily supply of prey cells [85].

Multiple studies have pointed out that the predation of phagotrophic bloom-forming species on their competitors or potential grazers may contribute to the success in monopolizing resources and forming dense, mono-specific blooms [113,118]. A recent bioassay suggests that phagotrophy or micropredation of *K. australe* might play a key role in the lethal effects on the marine animals rather than exotoxicity, especially at lower cell densities [73]. This may explain why many groups of autotrophic phytoplankton can grow rapidly and densely under a combination of light and nutrients in the laboratory but most of them cannot form monospecific blooms in the field [113]. Mixotrophic *Karlodinium* species also show a growth advantage in size [20,59]. For example, in *K. armiger* cultures with sufficient food, it was easy to reach a cell size of up to $9000 \mu\text{m}^3\cdot\text{cell}^{-1}$, and the mean biovolume was approximately twice the size ($2500\text{--}3000 \mu\text{m}^3\cdot\text{cell}^{-1}$) of non-fed cultures ($1200\text{--}1500 \mu\text{m}^3\cdot\text{cell}^{-1}$) [11]. Magnifying cell size may help to avoid part of predators specializing in smaller preys. More importantly, the large range of prey types, wide spectrum of prey size, and flexible nutritional modes of *Karlodinium*, such as *K. armiger* and *K. veneficum*, seems to make it a powerful competitor in marine plankton [4].

HABs of *Karlodinium* have been demonstrated to be highly related to mixotrophic predation. Adolf et al. suggested that prey abundance, especially the abundance of nanoplanktonic cryptophytes, was a key factor stimulating the formation of toxic *K. veneficum* blooms in eutrophic waters [86]. They also stated the key elements resulting in toxic

K. veneficum blooms, include (1) eutrophic environments, (2) co-occurrence of cryptophytes and *K. veneficum*, (3) a rapid response of cryptophytes to environmental opportunities (e.g., nutrient input) to bloom, and (4) mixotrophic predation of *K. veneficum* on cryptophytes, aided by allelochemicals (e.g., karlotoxins) produced by *K. veneficum* that improve prey capture and reduce grazing mortality of toxic strains [1].

Toxins and/or allelochemicals are involved in prey capture in this genus. Both *K. veneficum* and *K. armiger* were observed to immobilize preys by toxins, and then an ingestion process followed [41,58]. HABs of *K. veneficum* were assisted by karlotoxins and contributed to accumulations of toxic *K. veneficum* based on their relatively higher phagotrophic capacity compared to non-toxic cells. High densities of *K. veneficum*, when harmful blooms occurred, exhibited allelopathy to other co-occurred algae by suppressing their physiological activity and growth rates [19,88,120], which induced other microalgae species more favorable to being captured.

It was assumed that once the mixotrophic harmful algal population has reached bloom density, mixotrophic feeding may not play a key role because preys were significantly reduced [121]. However, cannibalism, the recently found nutrient mode in *K. veneficum*, may help in maintaining population levels after the bloom is formed by consuming the dead cells of their own species [49]. This may explain the unusual phenomenon that certain harmful algal blooms maintain high cell densities even when nutrients are exhausted [122].

Based on the significant role of mixotrophy in bloom formation and dynamics in general, many new factors should be taken into consideration when we attempt to prevent and control HABs caused by mixoplankton. For instance, elimination of inorganic nutrient loading may not work well for this type of bloom. Other than inorganic nutrients and hydrological conditions, factors such as dissolved organic matter and even co-occurring plankton species could contribute to the formation of these blooms. In addition, the elimination of HABs desiderates a healthy ecosystem and complex food web, because the more energy flows to higher trophic levels, the less energy mixotrophs can detain.

8. Perspectives for Future Investigations on the Mixotrophy in *Karlodinium*

8.1. The Ecophysiology of *Karlodinium* Under Global Changes

Mixotrophy constitutes an energy-saving and a compensatory mechanism to meet the cellular C demands, thereby gaining the necessary energy to cope with the abiotic stress such as cooling and warming under the ultraviolet portion of the spectrum [123]. This metabolic flexibility implies a competitive advantage under multi-driver conditions compared with strict phototrophic or heterotrophic metabolisms as it would allow them to acquire energy and nutrition from both sun and prey depending on the environmental conditions [123]. Thus, more studies ought to be carried out to evaluate the influences of global change on the ecophysiology of mixoplankton, such as *Karlodinium* species.

8.2. Molecular Basis of Phagotrophy-Relevant Genes in *Karlodinium* and Other Species

At present, we know few details about the molecular or genetic mechanisms involved in mixotrophs in modulating their photoauto- vs. phagohetero-trophic capabilities [114]. The environmental changes may play an important role in impacting the metabolic regulation of mixotrophs under stressful conditions, which need to be taken into account. It was demonstrated that the phagotrophy intensity of *Karlodinium* species increases under nutrient limitation [16,22,119]. Other factors such as prey density, prey species, nutrient concentration, water depth, and salinity were also observed to affect the switch and intensity of phagotrophy [16,21]. We have recently documented that the intensity of phagotrophy in *K. veneficum*, including cannibalism, changed with the growth stage [16]. However, how the change in phagotrophy intensity and the switch among feeding mechanisms are regulated at subcellular and genetic levels continues to be a “Blackbox”. Previous studies on the molecular and genomic mechanisms of phagocytosis were based on and limited to a small part of organisms from other groups, like the specialized phagocytotic cells of insects and mammals (e.g., macrophages), the amoebozoans *Dictyostelium discoideum*

and *Entamoeba histolytica*, and the ciliate *Tetrahymena thermophila* [124–128]. The molecular studies of phagocytosis in marine microalgae are rare and focus on non-dinoflagellates like the chlorophyte *Cymbomonas tetramitiformis* and chrysophyte *Ochromonas* sp. [129,130]. Considering the possibly early origin of phagotrophy and the relatively close evolutionary distances within protists, the knowledge obtained from these molecular studies on protists, ciliates in particular, should be a solid basis for generating testable hypotheses about the molecular mechanisms of phagotrophy in *Karlodinium*. Nevertheless, it is now the time to start investigations on the phagotrophy-relevant genes and their expression regulations, and the biochemical (e.g., enzymes, proteins, and signal chemicals) and cellular mechanisms in *Karlodinium*.

8.3. Energetics and Pathways Relevant to the Energy Metabolisms of Phagocytosis of *Karlodinium*

Once organic particles, as above mentioned, are ingested as foods into *Karlodinium* cells, these “particles” should be subsequently degraded and utilized via a series of energy metabolism-related pathways. In addition, ingestion of organic particles may exert an influence on other metabolic pathways. A transcriptomic analysis about the effects of light and prey availability on the global gene expression of a mixotrophic chrysophyte *Ochromonas* sp. demonstrated that the ingestion of bacterial prey resulted in prominent changes in major metabolic pathways of carbon and nitrogen [130]. With the very limited knowledge regarding to the molecular processes involved in phagotrophy of *Karlodinium*, we postulate that studies focusing on the energetics and energy metabolism pathways involved in phagocytosis may be a key step to comprehensively understand the molecular processes and ecological significance of phago-mixotrophy in *Karlodinium*.

9. Conclusions

Although *Karlodinium* as a group of small, unarmored dinoflagellates has been long overlooked, owing to the difficulty in identification, and the nutritional modes have been far less studied for most species of the genus, our current knowledge about the trophic modes of *Karlodinium* is worthy of a synthesis, as has been done in this review, to promote forward studies. *Karlodinium* species exhibit plastic and multiple trophic modes and switching between these modes allows *Karlodinium* species to use inorganic and organic, dissolved and particulate nutrients, and live and dead organisms, as nutrients, and even those contained in other individuals of the same species, via multiple instruments (e.g., peduncle and capture filament) and processes (e.g., engulfment, myzocytosis, etc.). *Karlodinium* species may not be able to survive well in any single mode, but the mixotrophic strategy certainly provides competitive advantages over other strictly autotrophic or heterotrophic competitors, by obtaining nutrients from multiple sources and killing competitors and even predators. In addition, the synergism among toxicity and allelopathy found at least in *K. veneficum* may also help to capture preys and avoid predation (Figure 2).

Mixotrophy, particularly phagotrophy, may have been a major contributor to the formation of harmful algal blooms and the achievement of a cosmopolitan distribution in species of *Karlodinium*, *K. veneficum* in particular, which thus deserves more in-depth investigations regarding the knowledge gaps that we have at least partly identified above.

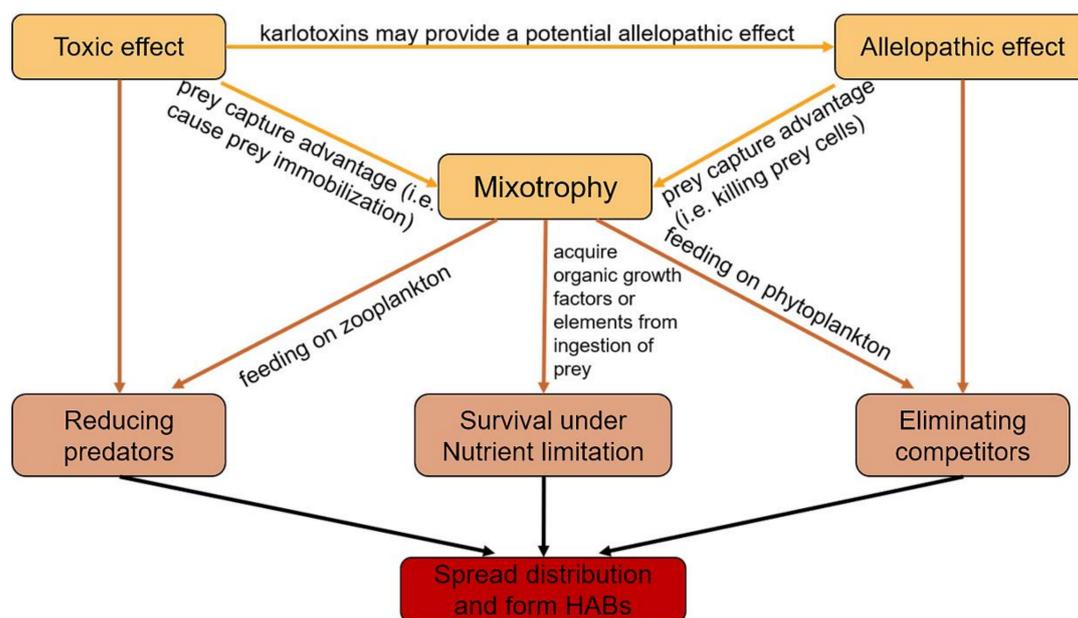


Figure 2. The relationship of phago-mixotrophy with toxicity and allelopathy of *Karlodinium*, and the implications for their global distribution and harmful algal blooms (HABs).

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