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Research review paper

RNA interference to combat the Asian tiger mosquito in Europe: A pathway from design of an innovative vector control tool to its application

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ABSTRACT

The Asian tiger mosquito *Aedes albopictus* is currently spreading across Europe, facilitated by climate change and global transportation. It is a vector of arboviruses causing human diseases such as chikungunya, dengue hemorrhagic fever and Zika fever. For the majority of these diseases, no vaccines or therapeutics are available. Options for the control of *Ae. albopictus* are limited by European regulations introduced to protect biodiversity by restricting or phasing out the use of pesticides, genetically modified organisms (GMOs) or products of genome editing. Alternative solutions are thus urgently needed to avoid a future scenario in which Europe faces a choice between prioritizing human health or biodiversity when it comes to *Aedes*-vectored pathogens. To ensure regulatory compliance and public acceptance, these solutions should preferably not be based on chemicals or GMOs and must be cost-efficient and specific. The present review aims to synthesize available evidence on RNAi-based mosquito vector control and its potential for application in the European Union. The recent literature has identified some potential target sites in *Ae. albopictus* and formulations for delivery. However, we found little information concerning non-target effects on the environment or human health, on social aspects, regulatory frameworks, or on management perspectives. We propose optimal designs for RNAi-based vector control tools against *Ae. albopictus* (target product profiles), discuss their efficacy and reflect on potential risks to environmental health and the importance of societal aspects. The roadmap from design to application will provide readers with a comprehensive perspective on the application of emerging RNAi-based vector control tools for the suppression of *Ae. albopictus* populations with special focus on Europe.

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1. Rationale for alternative vector control tools in Europe

The mosquito *Ae. albopictus*, also called the Asian tiger mosquito, is a vector for at least 22 human pathogens and poses a serious public health threat in large parts of the world. Originally from Asia, the arboviral vector became invasive and is still spreading around the world. In Europe, *Ae. albopictus* has been the vector for autochthonous chikungunya and dengue outbreaks. The first outbreak of those tropical infectious diseases occurred in Northern Italy in 2007 with >300 chikungunya patients. In 2010, the first autochthonous dengue transmission was detected in France and Croatia. Since then, arboviral outbreaks recurred in the Mediterranean countries. At present *Ae. albopictus* is established in 21 European countries with its northernmost population located in Germany (ECDC, 2022). As climate change and global transportation are further on the rise, the frequency and geographical range of *Ae. albopictus* and associated chikungunya and dengue outbreaks in Europe could also increase in the future (Oliveira et al., 2021).

To control the spread of dengue and chikungunya infections, new vaccines are currently under development, but there are challenges in vaccine development and licensing (Hucke et al., 2021). In the case of dengue, the first licensed vaccine is only recommended for seropositive individuals and second-generation dengue vaccines are not yet on the market (Wilder-Smith, 2020). Next to individual prophylactic measures against mosquito bites (e.g., long-sleeved clothes, repellents), the control of dengue and chikungunya vectors is currently the major line of arboviral risk reduction (Bellini et al., 2020). In Europe, the removal of mosquito breeding sites and larval control are common preventive measures, but their efficacy is limited by the inaccessibility to private properties during vector control operations (Reuss et al., 2020).

Several biological and chemical control tools against *Ae. albopictus* are available in Europe (Baldacchino et al., 2015; Benelli et al., 2016; Takken and van den Berg, 2019). Chemical insecticides are mainly applied against adult mosquitoes. Their use in Europe is granted under exceptional circumstances such as during a disease outbreak only (Bellini et al., 2014). However, insecticide resistance has been already reported in *Ae. albopictus* populations, for example from Italy and Vietnam (Kasai et al., 2019). Besides that, there are a number of larvicide products on the European market, with at least one formulation containing *Bacillus thuringiensis israeliensis* (Bti) (ECHA, 2021). Resistance against Bti has been reported in mosquitoes, but is not a widespread phenomenon (Suter et al., 2017). Though efficient against the vector species, Bti and chemical insecticides can have side-effects on non-target organisms (NTOs) (Boisvert and Boisvert, 2000; Moura and Souza-Santos, 2020). European regulations for the protection of biodiversity aim to phase out pesticides, and this would strongly limit the control options for *Ae. albopictus*.

Thus, new vector control approaches are urgently needed to complement our vector control toolbox, and to reduce the risk of *Ae. albopictus* transmitted arboviruses in line with Europe's biodiversity protection agenda and public health guidelines. Recently, the sterile insect technique (SIT), the incompatible insect technique (IIT), and transgenic approaches have been tested for vector control of *Aedes* mosquitoes (Morán-Aceves et al., 2021). These techniques are based on male sterilization by either gamma-ray irradiation or cytoplasmic incompatibility, or genetic modifications that either introduce dominant lethal genes or manipulate genes that alter biological processes such as fecundity, survival, and sexual determination of the offspring of genetically modified mosquitoes (GMM). Those techniques have in common, that modified males are released to field sites, mate with wildtype females and thereby suppress mosquito populations. The use of GMM's for a global vector control approach is slowed down by complicated regulation procedures (Mitchell and Bartsch, 2019). In Europe, the only used SIT approach is based on gamma-irradiated *Ae. albopictus* males with the purpose to reduce or eliminate remaining and inaccessible populations (Becker et al., 2022).

RNA interference (RNAi)-based control products may be an alternative to GMO or conventional pesticide in vector control. First results from North America, Australia, and Europe suggest that consumers differentiate among biotechnology solutions and may prefer topical RNAi-insect control to transgenic GMO insecticides (Shew et al., 2017). The proof-of-principle for RNAi-based control of *Aedes* mosquitoes was already shown by (van Ekert et al., 2014), but the full pathway from bioengineering an RNAi-based insecticide to its use in integrative vector control management has not yet been outlined.

In this paper, we review different aspects of RNAi-based vector control as an emerging approach to combat *Ae. albopictus* with a particular focus on its application potential in Europe. We describe a pathway from the development of RNAi approaches to their practical application in the context of regulatory compliance and integrated vector management (IVM) in Europe. According to our literature review on RNAi against *Aedes* mosquitoes (Supplementary data), we identified knowledge gaps (Fig. 1) regarding the relationship of RNAi to environmental and human health as well as the regulatory framework for its application and role in IVM. We then recommend priority experiments for the development of RNAi-based strategies targeting *Ae. albopictus*.

2. RNAi-based strategies for mosquito control

2.1. RNAi pathways in insects

Three RNAi pathways based on different types of small RNA molecules are triggered in insects (Zhou et al., 2008). The Piwi-interacting RNA (piRNA) pathway is triggered by RNAs created from long single-strand precursor RNAs due the so called "ping-pong" amplification mechanism resulting in molecules of 24–32 nts in length. The microRNA (miRNA) pathway has nuclear as well as cytoplasmic phases and is involved in the regulation of gene expression. The mature miRNAs are 22–23 nt RNA duplexes, derived from endogenously expressed stem-loop precursors that fold into characteristic imperfect hairpin-structures. Detailed descriptions of the molecular mechanisms underlying both pathways are given in several reviews (e.g., Cooper et al., 2019; Liu et al., 2019; Olson and Blair, 2015). The siRNA pathway is mostly cytoplasmic and triggered by long dsRNA, which can be either endogenous or exogenous (Claycomb, 2014; Ghildiyal and Zamore, 2009). It is evolved as a way to suppress viruses and transposons with a dsRNA replication form, but can also be induced by exposure to synthetic dsRNAs and is therefore the pathway used to control disease-vectoring mosquitoes (Fig. 2). The siRNA mechanism triggered by exogenous dsRNA is now well characterized in aedine mosquitoes and involves the cleavage of a long dsRNA molecule by the enzyme Dicer 2 (Dcr2) in association with the dsRNA binding protein R2D2 to form short interfering RNA (siRNA) duplexes 21–24 nts in length, which are loaded on the protein Argonaute 2 (Ago2) (Marques et al., 2010). This forms the core for the assembly of an RNA-induced silencing complex (RISC) that results in the degradation of the siRNA sense strand, leaving the antisense strand free to bind its complementary target, which is then cleaved by Ago2 (Matranga et al., 2005). Although this mechanism is broadly conserved, there are species-dependent differences reflecting the duplication and divergence of genes encoding the RNAi machinery. For example, there are two Ago2 paralogs in *Culex pipens* (Airs and Bartholomay, 2017).

2.2. Identification of suitable RNAi target genes in Aedes species

RNAi-based methods for the control of arbovirus-transmitting mosquitoes can be divided into those targeting larvae and those targeting adult insects (Fig. 2). The anticipated outcome of most RNAi strategies targeting larvae is death before or during pupation or the severe disruption of development (Airs and Bartholomay, 2017). In contrast, RNAi strategies targeting adult mosquitoes in some cases aim to disrupt feeding or olfaction, but in most cases aim for population

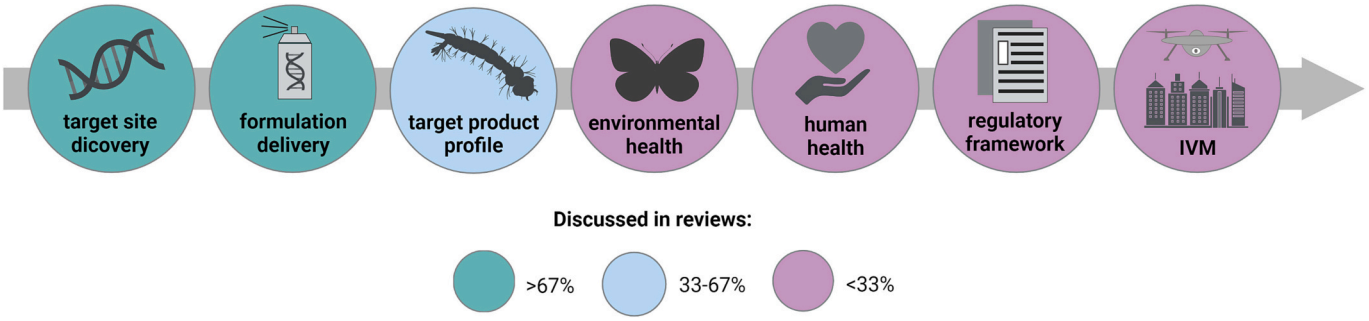


Fig. 1. The seven categories of knowledge that are crucial to develop, assess and implement RNAi-based vector control against *Aedes albopictus* in the context of regulatory compliance and integrated vector management (IVM) in Europe. The availability of information per category is indicated by the percentage of review articles, that appraised the different aspects of RNAi-based control methods targeting *Aedes albopictus*. The data reflect our analysis of the Web of Science database, discussing RNAi-based control for *Aedes*. Search term [Aedes AND RNAi AND control] has been entered at 12.03.2023 and resulted in 22 hits. Thirteen reviews fulfilled the inclusion criteria as stated in the Supplementary data.

control, either via SIT methods in which RNAi leads to the development of sterile males or male-dominated populations, or by the disruption of embryogenesis (Agarwal et al., 2022; Darrington et al., 2017; Hoang et al., 2016).

Larvicidal RNAi strategies in insects often target cell cycle regulation in an effort to induce apoptosis, reflecting the discovery of >400 dsRNAs that cause lethal knockdown by inducing apoptosis in *D. melanogaster* cells (Boutros et al., 2004). Accordingly, the IAP1 gene encoding a key inhibitor of apoptosis was targeted in *Ae. aegypti*, resulting in the rapid onset of mortality (Liu and Clem, 2011; Ocampo et al., 2013; Pridgeon et al., 2016; Puglise et al., 2016; Wang et al., 2012). Enzymes mediating chitin synthesis such as chitin synthases A and B have been identified as RNAi targets in *Aedes* larvae using either chitosan nanoparticles for formulation of orally delivered dsRNAs (Zhang et al., 2010) or in vivo expression dsRNAs in *Escherichia coli* added to the breeding water (Lopez et al., 2019). Further examples of target genes for RNAi in mosquitoes addressing different pathways and targets in different developmental stages are shown in Table 1. Up to 50% of *D. melanogaster* genes have orthologs in mosquitoes, suggesting this pool of dsRNAs could be used to select other mosquito targets (Zdobnov et al., 2002). If no known orthologous genes are available, possible RNAi target genes could be predicted by artificial intelligence or through large-scale RNAi screenings in model organisms like *Tribolium castaneum* (Knorr et al., 2021; Ulrich et al., 2015). Results could subsequently be transferred to other species (Mehlhorn et al., 2021).

Useful targets are constitutively expressed genes required for basic cellular functions such as cytoskeletal organization, ion transport, and intracellular trafficking. The suppression of tubulin and actin genes is lethal in many insects. In *Ae. aegypti* and other mosquitoes, larvae soaked in β-tubulin (Singh et al., 2013) or actin dsRNA develop an increased sensitivity to the Bt toxin Cry11Aa (Cancino-Rodezno et al., 2012). Vacuolar ATPase (vATPase) is a common target in insects because it is required for membrane proton translocation, although the efficacy of treatment depends on which subunit is targeted and the precise targeted region due to the presence of multiple paralogs and splice variants. The knockdown of proteolipid subunit (vATP-V0B) by injecting dsRNA into *Ae. aegypti* reduced survival, fecundity and fertility, and also the titer of dengue virus (Kang et al., 2014). In contrast, the knockdown of subunit A (vATP-A) did not cause lethality (Coy et al., 2012), although variation in monitoring periods limits the generalization of these studies. The inhibition of subunit B increased Cry11Aa toxin sensitivity in *Ae. aegypti* (Cancino-Rodezno et al., 2012) and the densovirus-mediated delivery of siRNA to silence vATPase subunit A significantly reduced the lifespan of *Ae. albopictus* (Gu et al., 2011). In the context of intracellular trafficking, RNAi suppression of coatomer proteins was lethal in *Ae. aegypti* because these are required for the trafficking of nutrient-containing vesicles in midgut cells (Isoe et al., 2011; Isoe et al., 2013; Zhou et al., 2011).

Several reports discuss the use of RNAi to disrupt host-seeking and feeding in adult mosquitoes through the suppression of olfactory processes. For example, the inhibition of the olfactory receptors OR8 and OR49 in *Ae. aegypti* leads to an increased probing and extended time to full blood meal engorgement (Won Jung et al., 2015). The inhibition of OR7 in *Ae. albopictus* also reduced the ability of insects to select preferential hosts (Liu et al., 2016). In other cases, feeding and digestion can be inhibited directly. For example, suppression of the aegyptin gene in *Ae. aegypti* inhibits blood meal uptake and reduces egg laying (Chagas et al., 2014), while the suppression of genes encoding digestive enzymes either cause death by nutrient starvation or the suppression of oogenesis (Isoe et al., 2009; Isoe et al., 2011; Isoe et al., 2013). Therefore, most RNAi strategies in adults target reproduction or development, aiming for population suppression. Sterile males can be produced by suppressing testis-specific genes required for sperm development such as zero population growth (Thailayil et al., 2011) whereas the targeting of testis-specific transglutaminase results in deficient sperm storage (Rogers et al., 2009). Several testis-specific genes were targeted simultaneously by feeding larvae on bacteria expressing different dsRNAs and only 8% of the adult males were fertile (Whyard et al., 2015). The suppression of genes with sex-dependent expression patterns, such as transformer 2, can also generate strongly male-biased populations (Hoang et al., 2016). Many other targets of RNAi strategies are developmental genes, such as catalase-2, which inhibits oocyte development in *Ae. aegypti*, and thus reduce egg numbers (Hansen et al., 2011) to genes expressed in pupal development such as single-minded (Mysore et al., 2014), also in *Ae. aegypti*.

RNAi experiments also bare the risk of off-target effects, either do due insufficiently specific siRNA/dsRNA triggers, or through the hybridization of siRNA with unintended mRNA leading to non-specific effects (Chen et al., 2021). To prevent the risk of non-specific off-target effects, strategies like the chemical modification of the siRNA guide strand at position 2 from the 5′ end or the pooling of multiple siRNAs are used (Neumeier and Meister, 2020). These techniques demonstrated that dsRNA with >80% sequence identity with their target gene will decrease the risk of specific off-target effects. Additionally, they found that dsRNA constructs with ≥16 perfectly matched base pair segments, and segments that are larger than 26 base pairs segments with 5–8 mismatches are also able to trigger RNAi machinery (Chen et al., 2021). Other options are the identification of possible target sequences with an identity to other mRNA's of 19 bp length by bioinformatics means (e.g., online tools like dsCheck, Deqor or *E*-RNAi) and exclude them and running control experiments in which, the target mRNA is knocked down through a second non-overlapping fragment (Mehlhorn et al., 2021).

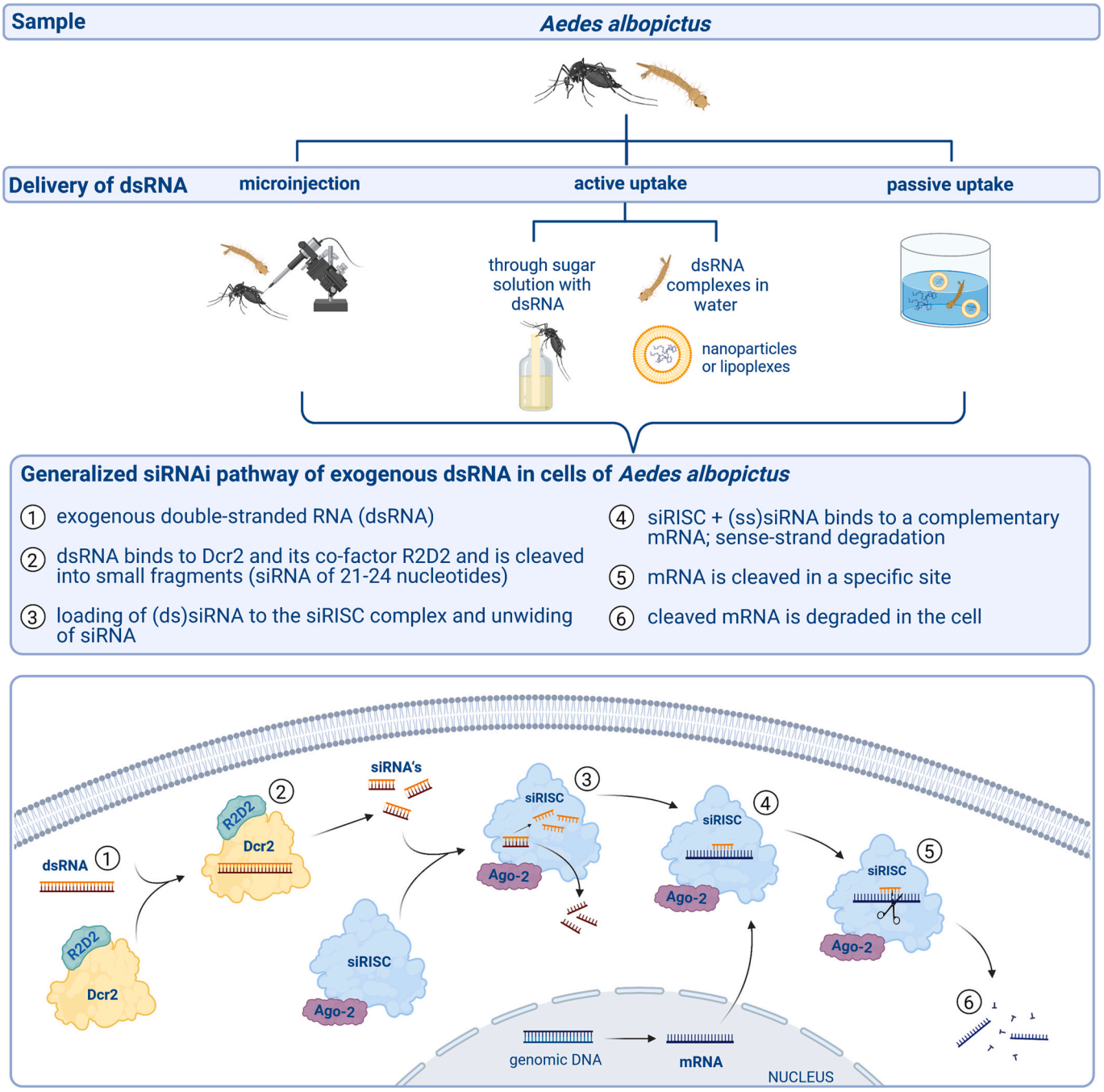


Fig. 2. Schematic overview about the possible application and mode of action of the siRNA pathway triggered by exogenous dsRNA in *Aedes albopictus* (modified after (Liu et al., 2019); created with Biorender.com).

2.3. Delivery and uptake of dsRNA to Aedes albopictus

The first hurdle is the administration of sufficient dsRNA to the mosquito larvae to ensure uptake and processing into siRNA. For small-scale experimental treatments, the common dsRNA delivery method is injection, which overcomes barriers such as the integument and allows the direct delivery of precise dsRNA doses (Munawar et al., 2020). For larger-scale experiments, dsRNA can be administered by soaking (Yu et al., 2013), dehydration/rehydration (Lopez-Martinez et al., 2012), or saturation using a micro-sprayer (Zhang et al., 2015), or by presenting dsRNA mixed in with the diet (Lin et al., 2017; Zhang et al., 2010). Laboratory methods such as injection and soaking are efficient on a small scale but impractical in the field. The efficient uptake of dsRNA is the main bottleneck in the control of mosquitoes by RNAi and this has been addressed by the development of formulations that encourage the ingestion of dsRNA or direct transfer across the larval cuticle (see section on formulations below). Assuming a sufficient dsRNA delivery has been achieved, the dsRNA has to be translocated from the gut to its target tissue. Early work in the nematode *Caenorhabditis elegans* indicated that dsRNA in the gut is translocated across the membrane of epithelial cells by the endocytic receptor SID2, but SID homologs are not present in all insects and even in those species with SID-like (SIL) proteins the inactivation of such proteins reduces the efficacy of RNAi in some species (Cappelle et al., 2016; Pinheiro et al., 2018; Tomoyasu et al., 2008; Yoon et al., 2016). Clathrin-mediated endocytosis (CME) appears to be the major mechanism for the uptake of both, long and short dsRNAs into

Table 1
Selected examples of target genes for RNA interference (RNAi) in mosquitoes addressing different pathways and developmental stages.

	Cell culture (C6/36) of *Aedes albopictus*	Eggs of *Aedes albopictus*	Larval Stages (L1-L4) living in water	Adult *Aedes albopictus*
Immune Response	**Argonaute-2 (Ago-2);** *increased replication of CHIKV*			**Argonaute-2 (Ago-2);** (McFarlane et al.2014) *increased replication of CHIKV*
	miR-281; *- decreased DENV-2 gRNA*			**miR-281;** (Zhou et al. 2014) *decreased DENV-2 gRNA*
	aae-miR-2940; (Slonchak et al. 2014) *- reduced metaloprotease level, leading to restricted WNV replication*			**Nonstructural 131 proteins (NS1-5);** (Magalhaes et al. 2019) *inhibition of Zika replication*
Development		**Netrin receptor Frazzled (frz);** (Clemons et al. 2011) *malformed ventral nerve cord during embryogenesis*	**Chitin synthase 1 and 2 (CHS1/2);** (Zhang et al. 2010) *disrupted chitin biosynthesis*	**Inhibitor of apoptosis protein 1 (IAP1); e.g.** (Pridgeon et al.2008) *mortality of injected adults*
			β-tubulin (β-tub); (Singh et al. 2013) *reduced growth and mortality of larvae*	
Reproduction		**Zero population growth (zpg);** (Thailayil et al. 2011) *spermless males*		**Male accessory glad-specific transglutaminase (MAG-TGase);** (Rogers et al 2009) *deficient sperm storage = reduced mating success*
		Transformer 2 (tra-2); (Hoang et al. 2016) *segregation distortion*		**Juvenile hormone acid methyl transferase (JHAMT);** (van Eckert et al. *2014*) *disabled egg and larval development*
Pesticide Susceptibility			**Inositol enquiring enzyme 1(Ire-1)/x-box binding protein (Xbp-1);** (Bedoya-Pérez et al. 2016) *hypersensitivity against Cry toxins*	**Protease m1 Zinc metalloprotease;** (Zou et al. 2016) *increased deltamethrin susceptibility*
			Mitogen activated protein kinase p38 (MAPK p38); (Cancino-Rodezno et al. 2010) *hypersensitivity against Cry toxin*	
Host Seeking and Feeding				**Olfactory receptors (OR8; OR49);** (Won Jung et al. 2015) *increased stylet probing, extended time for full engorgement*
				Olfactory receptor (OR7); (Liu et al. 2016) *reduced ability to seek hosts*
				Aegyptin; (Chagas et al. 2014) *reduced sensing of oviposition attractants*
				Odorant binding protein 1 (OBP1); (Pelletier et al. 2010) *increased probing time, smaller blood meals, reduced feeding success*

Legend
ways of RNA delivery
by injection
by nanoparticles or lipoplexes (ingestion by larvae or direct to cellculture)
delivery by soaking

For each pathway we selected four representative examples. Most of the studies were conducted in *Aedes aegypti*. Ways of RNA delivery are depicted by one of the three icons listed in the legend. This additional information points out, that most studies are done via microinjection and that there is a lack of using other, more field-applicable techniques (e.g., nanoparticles). The analyzed target gene plus reference study are in bold whereas the "treatment effects" (e.g., hypersensitivity) are in italics (Bedoya-Pérez et al., 2013; Clemons et al., 2011; Magalhaes et al., 2019; McFarlane et al., 2014; Pelletier et al., 2010; Pridgeon et al., 2008; Slonchak et al., 2014; Zhou et al., 2014; Zou et al., 2016).

insect cells, including hairpin dsRNA, as demonstrated by the use of specific inhibitors such as chlorpromazine or bafilomycin A1 to inhibit clathrin protein assembly (Da Xiao et al., 2015; Saleh et al., 2006; Yoon et al., 2016; Yoon et al., 2017) and the knockdown of CME-related genes (Cappelle et al., 2016; Pinheiro et al., 2018; Saleh et al., 2006; Wynant et al., 2014). The primary mechanisms for the uptake of naked dsRNA appear to be CME and to an extent SIL-dependent whereas macropinocytosis and phagocytosis may occur when the dsRNA is encapsulated in carriers or within living cells. Interestingly, CME appears to require at least one open dsRNA end because hairpin RNAs are taken up by CME whereas so called "paperclip" dsRNA structures with closed ends, are able to induce RNAi even when CME is inhibited, suggesting that those structures taken up via a clathrin-independent pathway (Abbasi et al., 2020).

2.4. Formulations for field applications of dsRNA

The field application of RNAi to control mosquitoes requires a formulation that protects the dsRNA from environmental degradation, concentrates the agent in the vicinity of the target and, preferably,

enhances its uptake by larvae and adults following ingestion (Adeyinka et al., 2020; Munawar et al., 2020; Rodrigues et al., 2022; Yan et al., 2021).

The formulation of dsRNA should also be suitable for application over wide areas, making sprayable products ideal. For RNA pesticide applications, foliar sprays, irrigation, and trunk injection associated with nanoparticles would be good choices to improve insecticidal efficiency (Yan et al., 2021). Mosquito larvae graze by filtering floating particles of food, including bacteria, fungi, and algae. The expression of dsRNA in transgenic microbes is therefore an ideal practical solution for the oral delivery of dsRNA to mosquito larvae and has been highly successful with live and heat-killed bacteria (Lopez et al., 2019; Taracena et al., 2019; Whyard et al., 2015), yeast (Hapairai et al., 2017; Hapairai et al., 2020; Mysore et al., 2017; Mysore et al., 2019a; Mysore et al., 2019b; van Ekert et al., 2014), and algae (Kumar et al., 2013). The delivery of dsRNA against vacuolar ATPase was achieved by the modification of *Ae. aegypti* densovirus (AeDNV) for the delivery of dsRNA to *Ae. albopictus* via the anal papillae following the spiking of rearing water (Gu et al., 2011). However, modified viruses are considered GMOs and GMO release into the environment comes with a high regulatory burden as well as problems in public acceptance (Schairer et al., 2021).

In addition, transfection reagents used in the laboratory to facilitate cell entry in vitro have also been tested for the delivery of dsRNA to mosquitoes, and those would probably be more suited for environmental release. For example, cationic liposomes that bind tightly to the negatively charged backbone of dsRNA have been used to silence the MAPK p38 gene and many others in *Ae. aegypti* (Cancino-Rodezno et al., 2010; Jiménez et al., 2012). Nanoparticles consisting of chitosan and dsRNA have also been used successfully in mosquitoes, including the silencing of two chitin synthase genes in *Anopheles gambiae* larvae following the failure of orally delivered naked dsRNAs targeting the same genes (Zhang et al., 2010). Chitosan is a strongly cationic polymer that forms complexes with dsRNA and therefore improves its stability, particularly against the highly alkaline environment and nucleases in the insect gut (Zhang et al., 2010; Zhang et al., 2015). In a spray formulation, the antimicrobial activity of chitosan would also protect the dsRNA from degradation by bacteria and fungi (Ke et al., 2021). The stability of chitosan/dsRNA nanoparticles has been improved by cross-linking chitosan to sodium tripolyphosphate, increasing the efficiency of delivery and therefore the magnitude of the knockdown effect against multiple genes and the effect on larval mortality (Dhandapani et al., 2019). Other nanoparticle formulations such as quantum dots and silica nanoparticles have been tested against mosquitoes, and a comparative study revealed that quantum dots were superior to the other formulations for the delivery of dsRNA against the *Ae. aegypti* genes SNF7 (vacuolar-sorting protein) and SRC (steroid receptor coactivator), probably reflecting their enhanced stability and efficient distribution in vivo (Das et al., 2015).

An ideal formulation would attract mosquito larvae, protect dsRNA molecules from the environment, be safe and biodegradable, and would release dsRNA in the gut but not in aqueous habitats such as artificial water containers and puddles, where larvae develop, perhaps by making the release mechanism dependent on a high pH. In addition, successful RNAi approaches require the presence of sufficient quantities of siRNA to achieve the required knockdown effect.

2.5. Efficacy of RNAi approaches

Even if an ideal formulation has been found for *Ae. albopictus*, the efficacy of RNAi varies according to many other factors, including the target gene (and region within the gene), the RNAi trigger (dsRNA, siRNA, hpRNA (hairpin RNA) or pcRNA (paperclip)), the insect species and target tissue, and the developmental stage, all of which influence the mechanisms of dsRNA uptake, processing, and inter- and intracellular trafficking.

For an efficient RNAi experiment it is recommended to design long dsRNA molecules of around 200–500 base pairs (bp) as the cellular uptake mechanism seems to require a minimal length of dsRNA (Vogel et al. 2019, Mehlhorn et al., 2021). For example, a study in *Diabrotica virgifera virgifera* (Western Corn Rootworm) showed that an uptake of 240 bp dsRNA targeting DvSnf7 (*Diabrotica vigifera* sucrose non-fermenting protein 7) was seen in midgut cells whereas a 21 bp siRNA was not (Bolognesi et al., 2012). Additionally, Saleh et al. (2006) showed a more efficient uptake of long dsRNA (up to 592 bp) than of short siRNA of around 21 bp.

The analysed target species is also important as studies have shown different outcomes in RNAi even for the same gene targeted. Rana et al. (2020), for instance conducted a comparative feeding analysis of an RNAi mediated knockdown of chitin synthase A in the lepidopteran species *Spodoptera litura* (tobacco cutworm), *Chilo partellus* (spotted stalk borer), *Plutella xylostella* (diamondback moth), and *Maruca vitrata* (bean pod borer). The analysed species showed significant variations in their sensitivity of chitin synthase A to RNAi. For example, there was a 20% reduction in the transcription level of chitin synthase A in *M. vitrata*, whereas the suppression in *P. xylostella* was 90%. The mortality was between 50% and 89%. Overall, RNAi mediated knockdown seems to be most efficient in coleopteran pests whereas experiments in Lepidotera and Diptera are more challenging (Cooper et al., 2019; Mehlhorn et al., 2021). One reason for the differences between species could be the stability of dsRNA which will be determined in part by the precise conditions of the gut (physicochemical conditions and the presence of microbes and enzymes) and the presence of nucleases. For example, several dsRNases were recently discovered in the larval gut of *Ae. aegypti* and inhibiting their expression increased the efficacy of RNAi significantly (Giesbrecht et al., 2020; Mehlhorn et al., 2021). Also, the host tissue is important because the efficacy of RNAi is much lower in the head and antennae than the rest of the body, with up to 8-fold higher doses of dsRNA required to target genes expressed in the anterior tissues, which is relevant for example when targeting genes involved in olfaction responses (Das and Dimopoulos, 2008). Overall, the preferred knockdown induced by a RNAi application in mosquitoes should result in >80% mortality and > 80% anti-viral effect (Table 2).

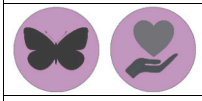
2.6. Evolution of resistances towards RNAi targets

As with all other methods for the control of insect pests and disease vectors, there is concern that the deployment of RNAi could lead to the emergence of resistant insect populations. A reason for resistance development could be an over-dose on dsRNA given to the organisms in the field leading to resistant alleles in subsequent generations (Choudhary et al., 2021). For instance, a study in L. *decemlineata* reported an increase of resistance against dsRNA targeting the V-ATPase subunit A gene of >11,100 fold (Mishra et al., 2021); (Choudhary et al., 2021). Other reasons can be mutations of target genes or core RNAi machinery genes as well as sequence polymorphism of target genes which can cause mismatches between dsRNA and mRNA (Yan et al., 2020). The likelihood of resistance could be reduced by designing siRNAs that target sequences containing codons for methionine and/or tryptophan residues, which each have a single codon and where all potential substitutions would be non-conservative. Similarly, the likelihood of resistance would be limited by delivering long dsRNAs that generate multiple siRNAs covering the same mRNA, although this runs the risk of cross-species effects in strongly conserved genes. The emergence of populations resistant to a specific siRNA could be rapidly countered by modifying the dsRNA to target the new mRNA sequence, or a different part of the same mRNA, or a different gene all together (Zhu et al., 2011).

2.7. RNAi effects on non-target organisms

One of the key advantages of RNAi as a control strategy is its specificity. Unlike most chemical insecticides, which also affect beneficial

Table 2
Target product profile for a hypothetical RNAi-based vector control product against *Aedes albopictus* in the European Union.

		Target Product Profile	Ideal Characteristics	Experimental Work needed
	#	**Formulation and Efficiency**		
	1	Target species	Effective against *Aedes albopictus* with larvae and adults as the main targets, eggs and pupae are secondary targets	Fundamental research on the food ecology of *Ae. albopictus*
	2	Target gene	Leading to population suppression by e.g. female sterility; anti-infectives through the suppression of uptake, development and transmission of pathogens	Identification of new conservative targets for anti-infectives that can stop the arboviral transmission chain
	3	Bioavailability	Uptake through larval feeding or adult sugar feeding; fast membrane-crossing and transfer through the midgut / crop barrier	In-depth studies of membrane-crossing of RNAi based vector control products through the midgut and crop barriers
	4	Efficacy	>80% mortality, >80 % anti-viral effect	Analysis of the spatio-temporal activity of RNAi based vector control products in mosquito cells and tissues
	5	Specificity	Species-specific, no development of resistance	Development of protocols for forced backcrossing experiments that would reveal rare occurrence of resistance
	6	Formulation	Chemically synthesized dsRNAs using e.g., cationic liposomes or chitosan; sprayable; not water-soluble; breaks off at high pH typical for midgut/crop of *Ae. albopictus*	Comparison of cationic liposomes and chitosan-based formulations for efficacy of larval and adult uptake
				Screening of formulations for water solubility, sprayability, pH stability, storage capability, and half-life in water
		Safety and Regulatory		
	7	Biosafety	No concern of RNAi-product (active ingredient) and its formulation for aquatic and terrestrial NTOs; no effects on human health	Inventory of aquatic microflora and non-target invertebrate species in *Ae. albopictus* infested containers in Europe in order to inform the design of RNAi products and NTO toxicity test batteries
		Manufacturing		
	8	Product stability	No need for cold chain; 1–2-year shelf life at room temperature	Analysis of product degradation under (sub)optimal storage conditions
	9	Environmental stability	Half-life in water that enables once weekly dosing	Efficacy testing under semi-field and field conditions
	10	Application	Spray; dispenser; applicable by private users and professionals	Testing the technical applicability by different user-groups
	11	Dosing frequency	7 days	Efficacy testing under semi-field and field conditions
	12	Concentration of active compound	Low	Development of protocols for detection of low amounts RNAi product in aquatic and terrestrial environment and biological tissues
		Scalability		
	13	Scalability	Simplified standard procedure for product production; user-attractive packaging design	Development of (simplified) standard procedures and quality indicators for production of RNAi based vector control products
	14	Usefulness for integrated vector control	Minimal interactions with commonly used chemical insecticides; positive interaction with Bt toxins	Toxicity testing of RNAi based vector control products in combination with insecticides currently used in Europe in acute and chronic bioassays with *Ae. albopictus*
		User Suitability		
	15	Cost	Affordable by local communities throughout Europe	Cost-benefit analysis in different countries and differing infestation stages of *Ae. albopictus*
	16	Information campaign/public relation/outreach campaign	Stakeholder group specific fair and accurate knowledge transfer formats on social-ecological risks and impact assessment, e.g. eco-friendly, non-GM profile, applicability	Promotion of awareness, building trust, perception of risk regarding the tiger mosquito and its diseases, ownership enhancement, and improvement of attitude towards such measures
				Development of knowledge transfer formats that depict citizens/local/regional/national authorities engaging in vector control

insects and, in some cases, also vertebrates, RNAi has the potential to target sequences that are unique to a given insect species, therefore preventing unwanted effects even if non-target species are directly exposed to the RNAi trigger (Airs and Bartholomay, 2017; Zhu and Palli, 2020). The likelihood of cross-species effects is related to the conservation of the target gene among different insect orders. Targets need to be conserved among populations of a species but vary from its sister species or in rare cases of genera where all species are pests among genera. A distinction can be made between genes that have undergone functional specialization for the niche occupied by mosquitoes (specific targets) and genes that fulfil basic cellular functions common to all insects (unspecific targets). Specialized genes, including those involved in host-seeking and blood-feeding, are likely to have diverged significantly between mosquitoes and other insects with different feeding habits, whereas genes involved in cell cycle regulation and core metabolism are likely to be much more strongly conserved. But even these ubiquitous genes with orthologs in all insects usually feature sufficient sequence variation to allow specific targeting, particularly in loop and coil regions beyond the key secondary structures that are required to maintain the overall protein fold.

For example, dsRNA constructs designed to target unique regions of the vacuolar ATPase subunit genes in *D. melanogaster, Manduca sexta, Tribolium castaneum*, and *Acyrthosiphon pisum* showed no evidence of cross-species effects (Whyard et al., 2009). In general, it is recommended to circumvent a sequence overlap of about 19–21 nts to avoid off-target effects (Bachman et al., 2013; Bolognesi et al., 2012; Whyard et al.,

2009). However, Powell and colleagues showed that between *Musca domestica* and *Delia radicum* a 15 nt sequence overlap was sufficient to induce off-target effects (Powell et al., 2017). Furthermore, mortality in *M. domestica* larvae after injection of 500 ng dsRNA targeting Diap1 (Drosophila Inhibitor of Apoptosis Protein 1) was 100% in 3 days and 5 days after injecting 250 ng dsRNA. *D. radicum* also showed 100% mortality after 3 days if 500 ng dsRNA were injected to 3rd instar larvae whereas injecting 250 ng showed a mortality of around 85% after 7 days (Powell et al., 2017). To further reduce the risk of such effects, software tools like dsCheck for estimating off-target effects caused by long dsRNA are available (Naito et al. 2005). Although, comprehensive testing for off-target effects is recommended, it should not be too challenging to design dsRNA constructs for the specific suppression of particular mosquito species, even when selecting highly-conserved target genes.

3. Bringing RNAi products to the European market

3.1. Regulatory framework of RNAi products in Europe

Bringing RNAi products to the European market will require further legal clarification. Depending on the type of RNAi product, different legislations may be applicable. Whereas plant-incorporated protectants via plant transformation follow the GMO legislation, externally provided RNAi products may or may not be regulated by this. Applications of microorganisms expressing RNAi are covered by the GMO legislation. However, liposome or chitosan-based RNAi formulations are not GMOs. In case GMOs are involved for their production, the challenge will then be to demonstrate that the final product is free of GMOs, non-viable and purified. RNAi products differ in regulation according to the purpose of application. RNAi products exogenously applied for the control of plant pests are classified as plant protection products and are regulated according to EC Regulation No. 1107/2009 (EC, 2009). In contrast, RNAi products for the control of mosquitoes are classified as biocides at the European market (EU, 2012).

Biocides are products to control unwanted organisms that are harmful to human or animal health or to the environment, or that cause damage to human activities. The Biocidal Products Regulation (BPR) of the EU (EU, 2012) and amendments provide rules to bring biocides to the EU market. The BPR makes a distinction between the active substance, i.e., the specific substance or microorganism that has an action on or against harmful organisms, and the biocidal product which is the formulated substance or mixture, in the form in which it is supplied to the target species. Biocidal products are often composed of mixtures of one or more active substances together with co-formulants such as stabilisers, preservatives, and colouring agents. In this case, the specific dsRNA will be the active substance, and when formulated in/with liposomes, chitosan, or other complexing reagents it is considered as the biocidal product. The approval of active substances takes place at EU level and the subsequent authorization of the biocidal products at Member State level, except for Union Authorizations. The European Chemicals Agency is the responsible authority for active substance approvals. Biocidal products are classified into 22 biocidal product-types, grouped in four main areas. RNAi products against mosquitoes would be designated Product-type 18: Insecticides, acaricides, and products to control other arthropods. As of today, no dsRNA-based biocide is or has been authorized in the EU yet. The Regulation's data requirements are specified for chemicals on the one hand and for microorganisms on the other hand. The fact that dsRNA does not fit into either class, is a challenge and needs an adapted regulatory framework.

3.2. Regulatory data requirements from the Biocidal Products Regulation

From a regulatory point of view, efficacy data, data on effects on NTOs including human health and on the environment as well as on the environmental fate of the product are mandatory to be included in an application dossier. Efficacy data are required to establish the benefit arising from the use of the RNAi product and must be balanced against the risks its use poses to human and the environment. Generally, efficacy data are generated from laboratory tests against the target organism, and/or (semi-) field trials. The mode of action needs to be explained including a problem description that is intended to be solved. Information on the development of resistance and appropriate management strategies is required as well as observations on undesirable or unintended side effects, e.g., on beneficial and other non-target organisms. An exposure assessment needs to be carried out for human and environmental populations. The effect on human and animal health is assessed using toxicological data (skin, eye test; studies testing mutagenicity, acute, repeated dose, and reproductive toxicity, carcinogenicity, etc.). Careful consideration of available information on toxicological properties may conclude on the need for some toxicological studies. Ecotoxicological studies include tests on aquatic organisms, including basic target taxa such as algae, daphnids, chironomids and fish. Furthermore, higher tier toxicity testing in aquatic mesocosm, the investigation of mechanism-specific effects such as endocrine and mutagenic potential and reactive toxicity should be applied. Terrestrial toxicity tests as well as tests on birds and mammals are required if the risk assessment indicates a concern. Information related to the fate and behavior of the active substance and its degradation products in the environment as well as other compounds in the formulations is needed in order to be able to assess the exposure to the environment. The BPR distinguishes between chemical biocides and microorganisms and mandates specific data requirements (Fig. 3). Although with some differences, the BPR requires similar studies and data compared to the plant protection products regulation.

3.3. Environmental impact of dsRNA

To develop an environmental impact and risk assessment, experiences in other fields of use of dsRNA are of interest. Crops engineered to express dsRNAs (genetically modified plants - GMP's) conferring resistance to pathogens or pest insects have reached the market although no dedicated guidelines have been developed for the risk assessment and regulation of RNAi-based GMPs at the international level (Papadopoulou et al., 2020). The authorization process of chemical pesticides in the EU requires applicants to perform a risk assessment considering food/feed and environmental safety aspects of living organisms or their derived food and feed products. Despite the fact that RNAi plants have received a favorable opinion (EFSA Panel on Plant Protection Products and their Residues (PPR), 2015) from the European Food Safety Authority (EFSA) for import and processing in the European Union, the *in planta* delivery of dsRNA via engineered crops is in conflict with the limited consumer acceptance of GMO's and falls under the same restrictions as other GMO plants (Arpaia et al., 2020; Christiaens et al., 2018). The applications of foliar dsRNA spray circumvent the use of dsRNA producing crops or microbes and therefore GMO related conflicts. The development of sprayable dsRNA for the control of mosquitoes in Europe benefits from corresponding approaches to combat pest insects. There is an ongoing debate on potential environmental impacts of dsRNA as addressed by the OECD Conference on Regulation of Externally Applied dsRNA-based Products for Management of Pests, which took place at the OECD in Paris, France, on 10–12 April 2019 (Romeis and Widmer, 2020). A major requirement will be feeding studies to assess whether the ingestion of dsRNA molecules poses a hazard to relevant non-target species. Non-target testing of chemical pesticides according to exposure risks is well established in Europe and guidelines are available. At the initial stage, only two species (e.g., daphnia (aquatic stage) or honey bees (adult stage)) are tested under worst-case exposure conditions. Additional screenings with other non-target species are only required if adverse effects above a certain threshold are detected for those species and unacceptable risk can thus not be excluded (Romeis and Widmer, 2020). Recent studies have addressed the fate of dsRNA in agricultural soils (Dubelman et al., 2014;

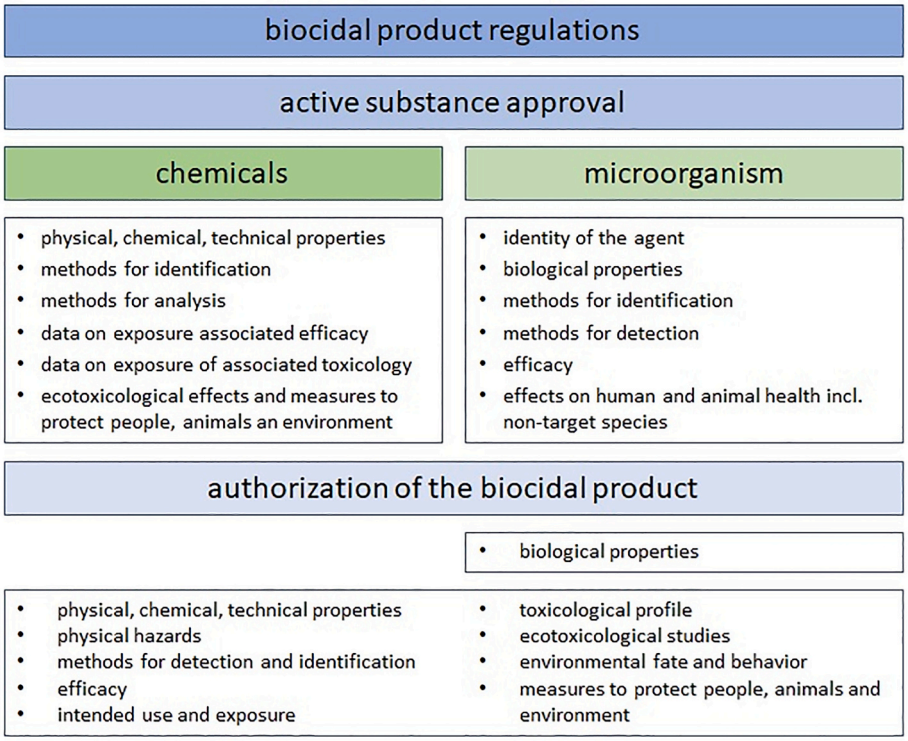


Fig. 3. Summarized data requirements from the Biocidal Products Regulation (EU, 2012) for active substances and their formulations via chemicals or microorganisms. The first step of the biocidal products regulation is the active substance approval, followed by the authorization of the biocidal product.

Parker et al., 2019), but there is little knowledge about the fate of dsRNAs in aquatic habitats. In aquatic microcosm and mesocosm studies, it has been shown that dsRNA rapidly degrades in the water column and dsRNA levels are non-significant in the sediment indicating that dsRNA is unlikely to persist in aquatic environments (Albright et al., 2017; Fischer et al., 2017). Authorities might request new toxicological studies if there is not enough information available on the toxicological properties of a substrate.

Consequently, a prerequisite for targeting mosquito larvae with dsRNA will be an ecological risk assessment with NTOs. An adequate spectrum of aquatic invertebrates and/or adult insects should be selected in order to test the uptake and effects of (non-)formulated dsRNA. In addition, a screening for off-target effects using bioinformatics tools is required, preferably already as part of the design of the target gene sequences in *Ae. albopictus* with low similarity to the genome of possibly exposed NTOs (Chen et al., 2021).

3.4. Integrative vector control management

An understanding of fine-scale mosquito population dynamics is essential to achieve greatest efficiency of RNAi-based larvicide and adulticide applications in the field. Their success strongly depends on the site selection and timing of treatment. The regional office of the World Health Organization (WHO) for Europe (Takken and van den Berg, 2019), the European VectorNet project (ECDC and EFSA, 2018), and the regional network 'Aedes Invasive Mosquitoes' (AIM) COST Action (Bellini et al., 2020) as well as initiatives in various EU member states (Kampen et al., 2015) collect data and develop sampling protocols for disease vectors. Their overall aim is also to provide and establish guidelines and recommendations for a cost-efficient and rigorous strategic surveillance system across Europe. Overall, a decrease in seasonal activity and abundances of *Ae. albopictus* from southern to northern Europe has been predicted (Pasquali et al., 2020) and might guide priority setting for an integrative vector control management. Risk mapping (linking high-resolution land use and land cover data to breeding sites) accounting for fragmentation of landscapes can assist with planning targeted and efficient control strategies (Baldacchino et al., 2017; Manica et al., 2016; McCormack et al., 2019; Petrić et al., 2014). Surveillance data from Northern Italy and a based-on mathematical model (Guzzetta et al., 2017) suggest that the focus for a cost-efficient larviciding of *Ae. albopictus* in urban areas should lie on most productive breeding sites and time period (in this case the month of July), especially if only one treatment is intended. A similar spatial and temporal 'hot spot' approach has been suggested for urban intervention sites in the USA (Unlu et al., 2016). In general, larvicides should have a longer lasting impact on densities and be more cost-beneficial when applied in early summer and during the warmer season. In contrast, adulticides should be preferred in autumn unless disease cases are reported early in the season (Trentini et al., 2018).

As stated in the 'Manual on the prevention of establishment and control of mosquitoes of public health importance in the WHO European Region' (Takken and van den Berg, 2019), larviciding, along with source reduction, can be applied at any stage of the establishment scenario, although an eradication is not feasible when the vector is established >25km^2. The trend of urbanization will further increase the number of artificial breeding sites available for container-breeding mosquitoes (Wilke et al., 2019) and will require the continuous help of citizens collecting data on, e.g., key containers in private spaces or adult sightings (Palmer et al., 2017; Werner et al., 2020) or applying larvicide control methods (Baldacchino et al., 2017; Guzzetta et al., 2017) at a local scale. Interventions for *Ae. albopictus* often apply a combination of source reduction, larvicide and adulticide treatment (Baldacchino et al., 2015; Bowman et al., 2016; Caputo et al., 2016), whereas assessments of the effectiveness of single-type treatments in the field are uncommon. Thus, RNAi-based larviciding and adulticiding would not be used as a stand-alone-tool, but is intended to improve the toolbox for vector control of *Ae. albopictus*.

3.5. RNAi-based vector control tools addressing European societal perspectives

To be applied in the European context, we argue that RNAi-based vector control tools should particularly address the distinct societal perspective incl. Attitudes, risk communication, and educational efforts (Adalja et al., 2016; Reuss et al., 2020). In general, any new technology should match the ethical, legal and social circumstances at the respective sites (Favia, 2015). The lack of knowledge on vector control and personal protective measures or on the level of awareness of the respective diseases is an important barrier for implementing community level interventions and personal protection against mosquitoes (Corrin et al., 2017). However, at the same time a good level of knowledge does not necessarily lead to good practices in implementing control measures. Cultural practices which are deeply ingrained in the local community, like water storage, might hinder people to implement measures (Hairi et al., 2003). Thus, people need to develop a sense of responsibility to accept and perform control measures on their properties (Elsinga et al., 2017). New approaches of citizen action where neighbors help neighbors showed promising effects in significantly reducing biting pressures by mosquitoes (Jordan et al., 2017). This concept works through respectful exchanges among scientists and residents and leads to trust and enhancement of ownership (Johnson et al., 2018). Good experiences with the involvement of citizens were made in the 19th century in Italy when fighting against malaria (Favia, 2015).

We may also learn from experiences with mosquito control using genetically modified mosquitoes (GMM). Residents were more likely to oppose GMM use if they had a low perception of the potential risks posed by diseases (Adalja et al., 2016). The perceived benefit-risk balance is a predominant variable to better understand the public acceptance (Amin and Hashim, 2015). Studies from France show that the perceived exposure to the vector appears to be one of the most significant reason of self-reported engagement in health-protective behaviors (Raude et al., 2012). In addition to that, trust in key players showed a direct influence on attitudes towards GMM of local people in Malaysia (Amin and Hashim, 2015) and the successful implementation of mosquito control measures targeting breading sites e.g., in Curacao (Elsinga et al., 2017). Thus, fair and accurate information for the general public regarding specific vector control tools aiming to fight vector-borne diseases is essential for their implementation.

4. Conclusion and recommendations

The dsRNA itself can be designed in a highly species-specific way (Chen et al., 2021). However, the application of dsRNA to control *Ae. albopictus* requires proper formulations, which facilitate dietary up-take by filter feeders or sugar soakers, protection from endogenous nucleases in their gut or crop, and translocation to the gut tissues (Munawar et al., 2020) as well as environmental stability to avoid rapid degradation. One option for the delivery of dsRNA is their expression in engineered bacteria or yeasts (Taracena et al., 2019; van Ekert et al., 2014), but the release of these GMOs, even when non-viable before their release into aquatic habitats, will raise public concerns and ethical debates. Therefore, we advocate for the use of chemically synthesized dsRNAs for mosquito control, which must be coated with adequate formulations such as nanoparticles, lipid droplets or chitin and its derivatives for application (Munawar et al., 2020). Ideally, these formulations, i.e., the natural or synthetic carrier materials should not have non-target effects independent of the RNAi treatment. Formulations can impose environmental hazards and require a corresponding risk assessment (Maletz et al., 2015; Romeis and Widmer, 2020). Consequently, before dsRNA spray can be introduced to the market, pre-market/prospective environmental risk assessments are required.

Based on these considerations, we recommend the development of a dsRNA-based product along with a target product profile (TPP), which fulfils 16 criteria for efficacy, safety, applicability, scalability in production, and acceptance on the market (Table 2). This TPP matrix for a hypothetical RNAi-based vector control product against *Ae. albopictus* in the European Union aims to align the product targets and characteristics with the needs of regulators and end-users and might be useful to guide product developers. RNAi-based vector control needs to meet also several societal and environmental needs such as being pesticide free, GMO free, and target specific and thus not affecting other organisms. The experiences from implementing previous control measures clearly show that integrating both, the societal and environmental needs and expertise is vital for a successful implementation. Thus, a transdisciplinary approach is needed for vector control that addresses stakeholders not only as an interest group but also as knowledge holders (Roberts and Thizy, 2022).

Future research should address both, the screening for highly efficient and *Ae. albopictus*-specific RNAi targets and the development of functional and non-hazardous formulations. More specifically, we used the TPP to list the experimental work of highest priority to close the most significant knowledge gaps.

The given outline from design to application of a hypothetical RNAi-based vector control product may provide stakeholders with a comprehensive perspective on the TPP and application of emerging RNAi-based vector control tools for the suppression of *Ae. albopictus* populations in the European Union.

Conflicts of interest

The authors declare no conflict of interest.

Declaration of Competing Interest

None.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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